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UNIVERSITY OF ALBERTA

A RECOMBINANT DNA-BASED APPROACH TO STUDIES OF SUCCINYL-CoA SYNTHETASE

by

DAVID G. RYAN ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING 1995

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Abstract

A new procedure for the production and purification of the subunits of succinyl-CoA synthetase was developed. The individual subunits of the *E. coli* enzyme were produced separately using a bacterial expression system. Both subunits accumulated as inclusion bodies in the bacterial cells in a manner that simplified their subsequent purification. The subunits purified from inclusion bodies reconstituted active enzyme with efficiencies similar to those achieved with subunits separated from the purified enzyme. This new procedure was employed for the production and purification of precursor forms of the α-subunit of both *E. coli* and rat liver succinyl-CoA synthetase. The precursor form of the rat liver α-subunit failed to accumulate in bacterial cells. Initiation of translation was adversely affected by the sequence encoding the precusor. Insertion of a leader sequence between the ATG initiator and the start of the open reading frame allowed the efficient production of a precursor form of the α-subunit.

Precursor forms of the α-subunit accumulated at significantly lower levels in bacterial cells than their corresponding mature forms. The lower levels of accumulation were the result of an increased sensitivity to proteolytic breakdown. It was suggested that the precursor forms of the α-subunit adopted loosely-folded translocation competent conformations in the bacterial cells. Like the mature subunits, precursor forms of the α-subunit accumulated in bacterial cells as inclusion bodies and were similarly purified. The

purified precursors were unable both to phosphorylate and to assemble with mature β-subunit. This was seen as evidence for the absence of a native-like conformation in precursor forms of the α-subunit. The results support a model in which the signal sequence is solely responsible for the attainment of the translocation competent state of a precursor.

cDNAs encoding two forms of the α-subunit of pig heart succinyl-CoA synthetase were isolated from a λgt11 library. One of the cDNAs, PHα108, contained a unique stretch of 108 nucleotides within its open reading frame that replaced a shorter 57 nucleotide section in PHα57. The amino acid sequence encoded by the 57 nucleotide stretch of PHα57 is conserved in other α-subunits. The mRNAs that gave rise to the two pig heart clones originated from a single gene and were the result of a mutually exclusive splicing event. The generation of the PHα108 message was found to involve atypical donor and acceptor splice sites. The dinucleotide AT was found in place of the preferred GT at positions +1 and +2 of its donor splice site. Furthermore, the common 3' acceptor splice site used to generate the two mRNAs is differentially utilized. The 108 sequence uses the dinucleotide AA rather than the preferred AG used by the 57 sequence at the this site.

The two cDNAs, PHα57 and PHα108, were combined with a cDNA encoding the β-subunit of the pig heart enzyme and expressed in bacterial cells. Production of the two subunits of each combination was coordinated by use of the translational coupling mechanism employed to coordinated the production of the corresponding bacterial subunits. The pig heart subunits were found to be insoluble

when produced at 37 °C. Production of the subunits at 20 °C alleviated this problem. Both of the pig heart cDNA combinations produced a GTP-specific enzyme. Extracts of the bacterial cells containing the two enzymes were fractionated by hydroxyapatite chromatography. The isoform produced by the β/α57 combination displayed an elution behaviour typical of that seen with the enzyme purified from pig hearts. The β/α108 enzyme eluted from the column at a phosphate concentration that was significantly lower than that required to elute the β/α57 enzyme. A preparation of succinyl-CoA synthetase from the heart tissue of a one week old piglet was found to contain an enzyme form that behaved similar to the β/α108 form on hydroxyapatite. These findings strongly suggest the presence of a α108-related protein in pig heart tissue. The position of the alternative sequence in the crystal structure of the enzyme suggests a possible regulatory role in catalysis.

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I am indebted to my supervisor, Dr. W. A. Bridger, for the opportunities and resources he provided. I would also like to thank Dr. W. T. Wolodko for advice and critical discussion throughout my studies. A special mention goes to Mr. Edward Brownie for his guidance in protein purification techniques. I am grateful to Dr. T. W. Lin for the frequent scientific discussion during my time in Dr. Bridger's laboratory.

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Table of Contents

SECTION I. INTRODUCTION

Chapter 1. Succinyl-CoA Synthetase, Fifty Years of Enzymology 2

I. Introduction 3

II. Metabolic and biosynthetic roles 3

A. Succinyl-CoA synthetase and the TCA cycle 3

B Other metabolic roles 4

C. Biosynthetic roles 7

D. Regulation of the enzyme 7

III. Biochemical characteristics 8

A. Sources of succinyl-CoA synthetase 8

B. Molecular weight and subunit structure 10

C. Substrate specificity 10

D. Polymorphism 11

E. Biogenesis and assembly 15

IV. The molecular reaction 17

A. The reaction mechanism 17

B. Kinetics and binding constants 21

C. Substrate synergism and conformational changes 22

D. Alternating sites cooperativity 28

V. Structure / function relationships 29

A. Primary structure of the subunits 29

B. The crystal structure 33

VI. Research aims 39

A. Biogenesis of the mammalian enzyme 39

B. Isoforms of the mammalian enzyme 40

C. Mutagenesis of the *E. coli* enzyme 41

References 42

SECTION II. BIOGENESIS AND ASSEMBLY

Chapter 2. Conditions for the Production, Purification and Refolding of Subunits of the *E. coli* Enzyme 54

I. Introduction 55

II. Materials and methods 57

A. Bacterial strains, plasmids and reagents 57

B. Expression and cell fractionation 57

C. Refolding of denatured subunits 59

III. Results 60

A. Production of the individual subunits 60

B. Subunits form insoluble aggregates 62

C. Purification of the subunits 62

D. Reconstitution of enzyme activity 64

E. Purification of the reconstituted enzyme 67

IV. Discussion 69

References 71

Chapter 3. Conditions for the production of precursor and mature forms of the mammalian α-subunit 73

I. Introduction 74

II. Materials and methods 75

A. Bacterial strains vectors and reagents 75

B. Vector construction and mutagenesis 76

C. T7-induced expression 78

D. RNA isolation 79

III. Results 79

A. The precursor fails to accumulate 79

B. The sequence encoding the signal peptide blocks expression 80

C. Inhibition is at the level of translation....84
D. The translational apparatus is functional....86
E. Initiation of translation is compromised....86
F. Secondary structure at the 5' end of the transcript....89
G. Increasing the distance from the TIR/AUG allows expression....90

IV. Discussion....93

References....95

Chapter 4. Folding and Assembly of the α-Subunit are Compromised in the Presence of a Signal Peptide....99

I. Introduction....100

II. Materials and methods....102
A. Bacterial strains, plasmids and reagents....102
B. Plasmid construction....102
C. Expression and purification of subunits....105
D. Refolding and autophosphorylation....105
E. Labeling and pulse-chase....106

III. Results....107
A. Precursor forms of the α-subunit are unstable *in vivo*....107
B. The precursor forms are unable to phosphorylate....111
C. The precursor fails to assemble with the β-subunit....114

IV. Discussion....116

References....118

SECTION III. MAMMALIAN ISOFORMS

Chapter 5. Alternative mRNA Splicing Produces a Novel Isoform of Pig Heart Succinyl-CoA Synthetase....122

I. Introduction....123

II. Materials and methods....124

A. Bacterial strains, enzymes and reagents....124

B. cDNA library screening....125

C. Genomic DNA libraries....125

D. Southern hybridization....126

E. Polymerase chain reaction (PCR)....126

III. Results....127

A. Isolation of cDNA clones encoding the α-subunit....127

B. Sequences unique to PHα108 are present in the gene....131

C. Cloning the α-subunit gene....133

D. Generation of the PHα108 message involves atypical splicing....135

IV. Discussion....138

References....142

Chapter 6. The Two Forms of the Pig Heart α-Subunit Support GTP-Specific Enzyme Activity....146

I. Introduction....147

II. Materials and methods....148

A. Bacterial strains, enzymes and reagents....148

B. Construction of expression plasmids....148

C. Expression....153

D. Enzyme fractionation....153

III. Results....155

A. Production of two forms of the pig heart enzyme....155

B. The two forms of the enzyme are GTP-specific....157

C. Separation of the two forms on hydroxyapatite.....159
D. Purification of the enzyme from pig hearts.....161

IV. Discussion.....164

References.....166

Chapter 7. Optimizing Production of the Pig Heart Enzyme in *E. coli*.....169

I. Introduction.....170

II. Materials and methods.....171
A. Bacterial strains, plasmids and reagents.....171
B. Vector construction.....171
C. Expression and western blotting.....174

III. Results.....174
A. Translational coupling in the bacterial genes.....174
B. The mammalian bicistronic unit.....176
C. The 28 kDa protein corresponds to the C-terminus of the β-subunit.....179
D. The 28 kDa protein is translated independent of the β-subunit.....179
E. Improving the translation initiation of the β-subunit.....183
F. The effect of growth at room temperature.....185

IV. Discussion.....187

References.....189

SECTION IV. CONCLUDING REMARKS

Chapter 8. Conclusions and Future Perspectives.....193

I. Conclusions.....194
A. Biogenesis and assembly.....194
B. Mammalian isoforms.....199
C. Expression of the mammalian sequences in *E. coli*.....204

II. Future perspectives.....206

References.....210

Appendices.....213

I. Bacterial strains and genotypes.....214

II. Oligonucleotides.....215

III. Media preparation.....216

IV. Chromatography.....217

List of Figures

Figure 1.1. Pathways utilizing succinate or succinyl-CoA.....5

Figure 1.2. Metabolic compartmentalization of succinyl-CoA synthetase activity.....14

Figure 1.3. The nonenzymatic reaction of succinyl-phosphate with CoA.....21

Figure 1.4. Reaction schemes for succinyl-CoA synthetase from *E. coli* and pig heart.....23

Figure 1.5. Conformational rearrangements in the phosphorylated enzyme.....27

Figure 1.6. Comparison of the amino acid sequences of the α-subunits of succinyl-CoA synthetase.....31

Figure 1.7. Schematic representation of the tetramer of succinyl-CoA synthetase from *E. coli*.....34

Figure 1.8. Schematic representation of the α- and β-subunits of the enzyme.....35

Figure 1.9. The role of αGlu_{208} in the phosphorylation of αHis_{246}.....38

Figure 2.1. Construction of plasmids for the production of subunits of succinyl-CoA synthetase.....58

Figure 2.2. Production of subunits of *E. coli* succinyl-CoA synthetase.....61

Figure 2.3. Fractionation of lysates of *E. coli* containing subunits of succinyl-CoA synthetase.....63

Figure 2.4. Purification of subunits from inclusion bodies.....65

Figure 2.5. Stoichiometry of assembly from denatured subunits....66

Figure 2.6. Purification of the active reconstituted enzyme....68

Figure 3.1. Plasmids and restriction site map of λSCS19-1....77

Figure 3.2. Expression of the open reading frame of λSCS19-1 in pT7-6....81

Figure 3.3. Examination of sequences encoding the signal peptide of the α-subunit....83

Figure 3.4. Transcription rates of T7-controlled genes....85

Figure 3.5. Translation of β-lactamase in cells containing pTZ19 plasmids....87

Figure 3.6. The ability of the *lac*Z TIR to mediate expression....88

Figure 3.7. Prediction of mRNA secondary structure....91

Figure 3.8. The effect of a leader sequence on the translation of λSCS19-1....92

Figure 4.1. Schematic illustrating the construction of expression plasmids for the mature subunits of the enzyme....103

Figure 4.2. Schematic illustrating the construction of expression plasmids for the precursor forms of the α-subunit....104

Figure 4.3. Production of mature and precursor forms of the α-subunit of pig heart and *E. coli* succinyl-CoA synthetase....108

Figure 4.4. The stability of subunits in bacterial cells....110

Figure 4.5. Phosphorylation of the two forms of the α-subunit....112

Figure 5.1. Alignment of the three groups of cDNAs for the α-subunit....128

Figure 5.2. Sequences of cDNAs encoding the α-subunit of pig heart SCS 130

Figure 5.3. PCR amplification of the α-subunit gene from porcine DNA 132

Figure 5.4. Hybridization of genomic clones to oligonucleotides specific for the alternative exons 134

Figure 5.5. Restriction endonuclease mapping of the overlapping clones 136

Figure 5.6. Alternative splice pathways and exon-intron splice sites for PHα57 and PHα108 137

Figure 6.1. Construction of an expression plasmid for production of pig heart succinyl-CoA synthetase I 150

Figure 6.2. Construction of an expression plasmid for production of pig heart succinyl-CoA synthetase II 151

Figure 6.3. Construction of an expression plasmid for production of pig heart succinyl-CoA synthetase III 152

Figure 6.4. Production of subunits of pig heart succinyl-CoA synthetase in *E. coli* 156

Figure 6.5. Succinyl-CoA synthetase activity in lysates of *E. coli* expressing pT7 β/α57 and pT7 β/α108 158

Figure 6.6. Elution profiles of the two isoforms from hydroxyapatite columns 160

Figure 6.7. Demonstration of a 108-related isoform in purified preparations of pig heart succinyl-CoA synthetase 163

Figure 7.1. Construction of expression plasmids for production of subunits of mammalian succinyl-CoA synthetase 173

Figure 7.2. Translation of the genes for *E. coli* succinyl-CoA synthetase 175

Figure 7.3. Expression of pig heart sequences in the bicistronic pT7 202 system 178

Figure 7.4. Analysis of fusion proteins of the pig heart β-subunit 180

Figure 7.5. The 28 kDa protein arises by translation initiation from within the open reading for the β-subunit 182

Figure 7.6. Mutagenesis of the two translation initiators and its effect on subunit levels 184

Figure 7.7. The effect of growth temperature on the solubility and yield of active enzyme 186

Figure 8.1. Model of a precursor form of the α-subunit of succinyl-CoA synthetase 198

Figure 8.2. Position of the alternatively spliced sequence in the α-subunit 203

Figure 8.3. Part of the active site of *E. coli* succinyl-CoA synthetase 207

List of Tables

Table 1.I. Characteristics of succinyl-CoA synthetase.....9

Table 1.II. Exchange reactions of succinyl-CoA synthetase.....18

Table 1.III. Kinetic constants of succinyl-CoA synthetase.....24

Table 4.I. Refolding and assembly of the two forms of the α-subunit.....115

Abbreviations

Å	angstrom
A_{600}	absorbance at 600nm
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
bp	base pair
CoA	coenzyme A
DTT	dithiothrietol
EDTA	ethylenediaminetetraacetate
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
GuCl	guanidinium chloride
IPTG	isopropyl-β-D-thiogalactopyranoside
ITP	inosine 5'-triphosphate
kbp	kilobase pairs
kDa	kilodaltons
NDP	ribonucleoside 5'-diphosphate
NMR	nuclear magnetic resonance
NTP	ribonucleoside 5'-triphosphate
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
P_i	inorganic phosphate
PMSF	phenylmethylsulfonylflouride
PRR	proton relaxation rate
PVDF	polyvinylidene difluoride

RBS	ribosomal binding sequence
S/D	Shine Dalgarno sequence
SCS	succinyl-CoA synthetase
SDS	sodium dodecyl sulfate
TCA	tricarboxylic acid cycle
TIR	translation initiation region
Tris	tris-(hydroxymethyl)aminomethane
UTP	uridine 5'-triphosphate

Section I

Introduction

Chapter 1

Succinyl-CoA synthetase, fifty years of enzymology

I. INTRODUCTION

Succinyl-CoA synthetase has been the subject of several reviews [1-4]. The time at which the most recent of these reviews [4] was published is significant in that it marks a turning point in research on the enzyme. In April of 1984, a report on the crystallization of the *Eschericia coli* enzyme was made [5]. One year later the genes encoding the *E. coli* enzyme were identified, providing the first primary structure of succinyl-CoA synthetase [6]. This information was to prove invaluable in solving the crystal structure. Over-expression of the *E. coli* genes produced the quantities of enzyme necessary for further structural work [7]. The crystal structure of the *E. coli* enzyme at 2.5 Å resolution was published this year [8]. Combined, these two areas of research have catapulted succinyl-CoA synthetase into a new era of molecular enzymology.

II. METABOLIC AND BIOSYNTHETIC ROLES

A. SUCCINYL-COA SYNTHETASE AND THE TCA CYCLE

Attention was first drawn to the enzyme from studies on the oxidative decarboxylation of α-ketoglutarate to succinate. It had previously been observed that this part of the tricarboxylic acid (TCA) cycle was accompanied by esterification of inorganic phosphate [9,10]. Kaufman [11] established that the oxidation proceeds through the formation of an intermediate, succinyl-CoA. He proposed a two step mechanism to describe the complete sequence of events. The

formation of succinyl-CoA and its subsequent conversion to succinate were found to result from two separate enzymatic activities, those of α-ketoglutarate dehydrogenase and succinyl-CoA synthetase, respectively [12]. Furthermore, enzyme preparations capable of esterification also liberated inorganic phosphate from ATP if both CoA and succinate were present [11]. Succinyl-CoA synthetase was identified as being responsible for the esterification and liberation of phosphate [12,13]. These early experiments provided us with an accurate description of the reaction catalyzed by the enzyme and firmly established its position within the TCA cycle (Fig. 1.1).

Succinyl-CoA synthetase (EC 6.2.1.4 [GDP specific], EC 6.2.1.5 [ADP specific]) is frequently referred to as succinic thiokinase [14]. The enzyme couples the nucleoside diphosphate-dependent conversion of succinyl-CoA to succinate with the esterification of inorganic phosphate by the following reaction:

$$\text{Succinyl-CoA} + \text{NDP} + \text{Pi} \overset{Mg^{2+}}{\rightleftharpoons} \text{Succinate} + \text{NTP} + \text{CoA}$$

where NDP and NTP are purine ribonucleoside 5'-diphosphate and 5'-triphosphate, respectively. The reaction can be viewed as an interconversion of two forms of high energy bond, the thioester of succinyl-CoA and the phosphoanhydride of NTP.

B. Other Metabolic Roles

Succinyl-CoA synthetase serves important roles in both forward and reverse directions of the reaction. In the forward direction as shown above, the enzyme provides a key intermediate,

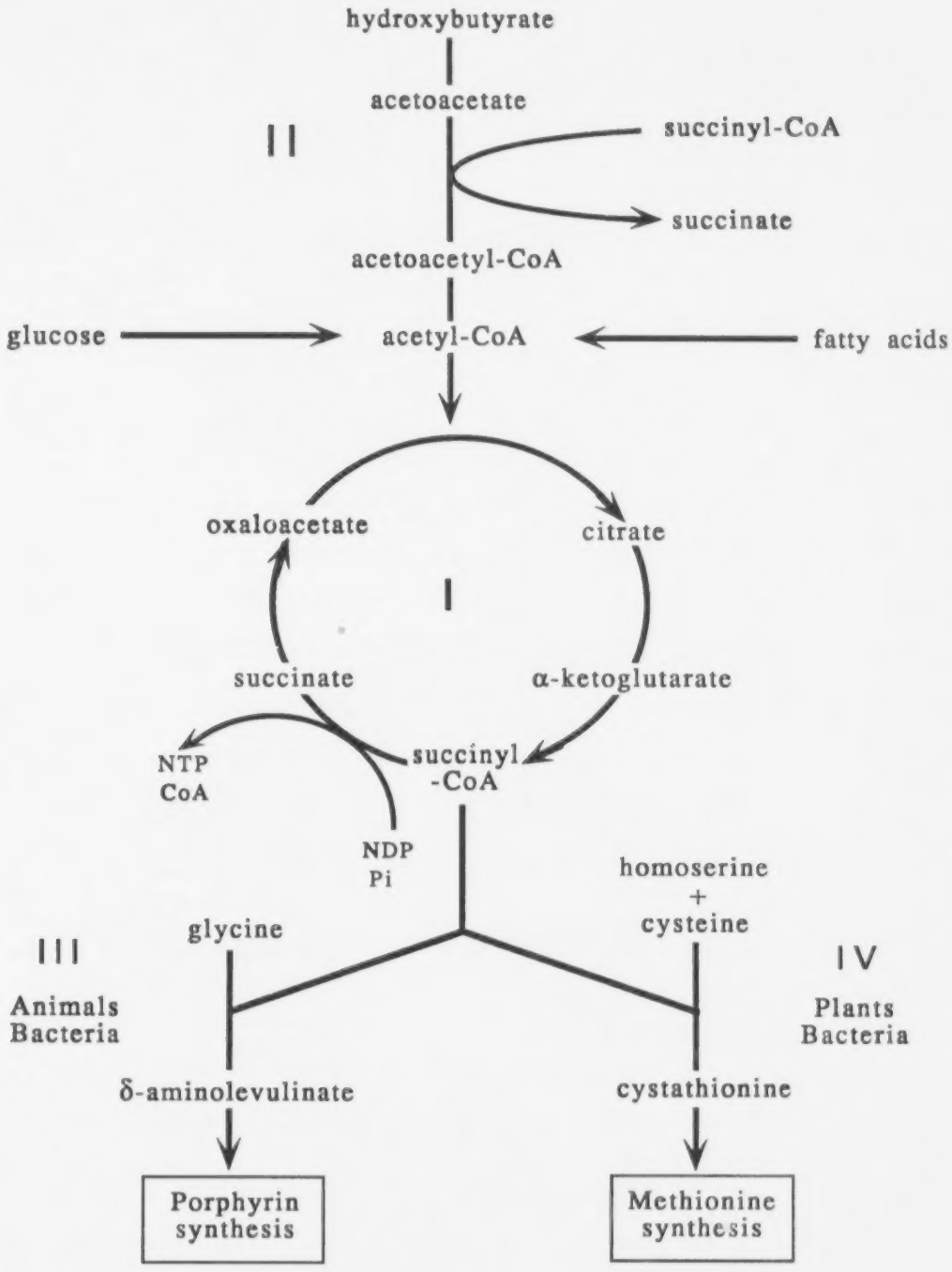

Figure 1.1. Pathways utilizing succinate or succinyl-CoA. Roman numerals denote the following metabolic and biosynthetic pathways: I, citric acid cycle; II, ketone body metabolism; III, porphyrin synthesis; IV, methionine synthesis. Taken from Stryer, 1988 [18].

succinate, to the TCA cycle (Fig. 1.1 [I]). In this direction it also carries out the only "substrate-level phosphorylation" event of the cycle. This is undoubtedly its primary function in most organisms. Succinyl-CoA synthetase plays an analogous role in the metabolism of Trichomonad flagellates. These protozoans lack mitochondria and carry out a fermentative form of metabolism [15]. Acetyl-CoA resulting from glycolysis of carbohydrates is converted to acetate. The enzyme responsible for this, acetate:succinate CoA-transferase [16], transfers the CoA from acetyl-CoA to succinate. The succinyl-CoA thus produced is recycled back to succinate by succinyl-CoA synthetase while the energy of the thioester bond is captured by substrate-level phosphorylation.

Succinyl-CoA produced at the expense of NTP in the reverse direction is important for ketone body metabolism. The metabolic activation of ketone bodies depends on succinyl-CoA [17]. In mammals, ketone bodies such as acetoacetate and β-hydroxybutyrate are produced by the liver when acetyl-CoA accumulates beyond its capacity to be oxidized by the TCA cycle [18]. Some tissues, such as heart, routinely derive much of their metabolic energy from the oxidation of these compounds. Other tissues such as the brain and skeletal muscle increase their utilization of ketone bodies when glucose levels are low. The ketone bodies are first converted back to acetyl-CoA in these tissues. Succinyl-CoA:3-ketoacid CoA transferase [19] catalyzes the crucial step in this conversion, the transfer of CoA from succinyl-CoA to acetoacetate (Fig. 1.1 [II]). High levels of succinyl-CoA must be maintained for continued utilization of ketone bodies [20]. It has been suggested that succinyl-CoA synthetase

accomplishes this by recycling the succinate produced in the transferase reaction [17].

C. Biosynthetic roles

Succinyl-CoA is an essential anabolic precursor [18]. This role is best illustrated by the observation that anaerobic organisms unable to oxidize acetate via the TCA cycle still require succinyl-CoA synthetase activity for biosynthetic functions [21]. Levels of succinyl-CoA synthetase are, in fact, repressed 10-fold in *E. coli* grown aerobically on glucose, conditions of high glycolytic activity [22]. The enzyme is, therefore, regarded as serving a predominantly biosynthetic role in the enteric bacteria [23].

As an anabolic precursor, succinyl-CoA serves both porphyrin and cystathionine synthesis [18]. Porphyrins are a class of tetrapyrrole compounds that include heme, chlorophyll, phycobilin and cobalamin. All porphyrins are derived from the common precursor, δ-aminolevulinic acid [18]. In bacteria and animals, this precursor is formed from succinyl-CoA and glycine (Fig. 1.1 [III]). Hence, the pathway is often referred to as the succinate-glycine pathway. Cystathionine is an essential precursor for methionine synthesis. In plants and bacteria, cystathionine is formed from homoserine and cysteine [18]. Succinyl-CoA is converted to succinate and CoA during the initial steps in the pathway (Fig. 1.1 [IV]).

D. Regulation of the enzyme

Allosteric regulation is expected in an enzyme with such a diversity of metabolic and biosynthetic roles. Succinyl-CoA synthetase from *E. coli* was tested with a variety of nucleotides,

coenzymes, and intermediates of both the TCA cycle and porphyrin biosynthesis [24]. No inhibition of an allosteric nature was detected. The enzyme from *Rhodopseudomonas spheroides* , a photosynthetic bacterium, was shown to be inhibited by protoporphyrin and hemin [25]. Similar control by products of porphyrin synthesis has been suggested for the mammalian enzyme [26]. Inhibition by hemin and glutathione were observed in the enzyme from soybean callus [27]. This last case is unusual since synthesis of δ-aminolevulinic acid in plants does not use the succinate-glycine pathway [18].

III. BIOCHEMICAL CHARACTERISTICS

A. Sources of succinyl-CoA synthetase

The most active preparations of succinyl-CoA synthetase have been obtained from the bacterium, *E. coli* [24,28-31] and from pig heart tissue [32-34]. Consequently these are the best characterized examples of the enzyme. Purification of the bacterial enzyme has been facilitated by the fact that the levels of the enzyme are elevated in cells grown on succinate medium [22]. Improvements in the purification procedures for the mammalian enzyme have been described [35,36]. Although similar in their catalytic properties, several significant differences are found between these two enzyme species (Table 1.I). Succinyl-CoA synthetase has also been purified from pigeon pectoral muscle [37], rat liver [38], a variety of plant tissues [27,39-42] and from the photosynthetic bacterium, *R. spheroides* [43].

Table 1.I

Characteristics of succinyl-CoA synthetase

	E. coli **enzyme**	**Pig heart enzyme**
Molecular mass	140 kDa [28,29]	75 kDa [33]
Quaternary structure	Tetramer $\alpha_2\beta_2$ [48]	Dimer $\alpha\beta$ [48]
Subunit size	α = 29.5 kDa β = 41.0 kDa [47]	α = 32 kDa β = 42 kDa [35]
Nucleotide specificity	"Relaxed" ATP>GTP>ITP [52]	"Restricted" ATP or GTP [61]
Genetic polymorphism	None reported	Yes [26,61]
Biogenesis of subunits	Synthesized as mature subunits [6]	Synthesized as precursors [75,76]
Subcellular location	Cellular cytosol	Mitochondrial matrix

B. Molecular mass and subunit structure

Molecular masses of 140 - 160 kDa were estimated for succinyl-CoA synthetase from *E. coli* [24,28,29]. The values obtained for the pig heart enzyme, 70 - 80 kDa, are roughly half those of the bacterial enzyme [33,35,36]. The enzyme from other eukaryotic species including the yeast, *Saccharomyces cerevisia,* is similar in size to the pig heart form [38,44]. In addition, all the gram-positive bacteria examined contain this smaller form [44,45]. The only organisms found to contain an enzyme comparable in size to the *E. coli* form are the gram-negative bacteria [44,45,46].

Both the *E. coli* [47] and the pig heart [35,36] enzymes were found to contain two different subunits. The smaller subunit was designated the α-subunit and the larger one the β-subunit. Molecular masses of 29.5 kDa for the α-subunit and 40 kDa for the β-subunit of the *E. coli* enzyme were reported [47]. Estimates of 32 kDa and 42 kDa were given for the subunits of the pig heart enzyme [35]. The amino acid composition of the native enzyme was consistent with there being equimolar amounts of the two subunits [3]. Thus, taking account of the observed molecular weight estimates, it was established that the active form of the *E. coli* enzyme is a nondissociating $\alpha_2\beta_2$ tetramer while the pig heart enzyme is a nonassociating αβ dimer [48].

C. Substrate specificity

Among the naturally occurring carboxylic acids tested, the enzyme exhibits a high degree of specificity for succinate [13,28,39]. Low rates of thioester formation were observed with malate at high

(91 mM) concentrations [49]. The organic acids, α-methylsuccinate and α-methylenesuccinate, are good substrates giving initial rates of acyl-CoA formation comparable to that measured with succinate [50]. Of the divalent metal ions tested, Mg^{2+}, Mn^{2+} and Co^{2+} were active in decreasing order of effectiveness [28,41,42]. Dephospho-CoA can replace CoA as a substrate for both the *E. coli* [51] and the pig heart [33] enzymes while the analogue, 4-phosphopantetheine is a poor substrate for the *E. coli* enzyme [49].

Succinyl-CoA synthetase from *E. coli* [52], *R. spheroides* [43] and a variety of other bacterial species [46,53] will accept either ATP, GTP or ITP. With the enzyme from *E. coli*, the order of effectiveness as judged by high V_{max} and low K_m is ATP > GTP > ITP [52]. In contrast, the eukaryotic enzymes appear to be more discriminating in their use of nucleotides. Succinyl-CoA synthetase from plants [27,39-42] and yeast [54] exhibits specificity for ATP. The mammalian enzymes that have been purified were found to be specific for nucleotides with the 6-oxo substitution, such as GTP or ITP [55]. GTP can be replaced by 8-azaGTP [32] or 6-thioGTP [33] in the pig heart enzyme. More recent studies in a variety of animal species including mammals have demonstrated the presence of distinct ATP- and GTP-specific enzymes (see below).

D. Polymorphism

Baccanari and Cha [56] detected multiple forms of pig heart succinyl-CoA synthetase by isoelectric focusing. These forms, however, were found to be interconvertible following incubation with the appropriate substrates or with sulfhydryl reducing agents

[56,57]. Thus, the differences did not appear to be related to variations in amino acid sequence. Two distinct GTP-specific enzymes were seen in mouse liver mitochondria [26]. The two forms could be distinguished by their heat inactivation profiles, pH responses, and by their behaviour to cellulose acetate electrophoresis. One of these enzyme forms was elevated 300 % under conditions of increased porphyrin synthesis whereas the other remained constant. These observations suggest the involvement of a GTP-specific isoform in the synthesis of heme and related compounds.

Although mammalian succinyl-CoA synthetase had been characterized as GTP-specific [55], examples of ATP-specific enzymes were reported in other animal species [55,58]. It is interesting that the original work with preparations of the enzyme from mammalian muscle was carried out using ATP and ADP as substrates [11-13]. In more recent studies, both ATP- and GTP-specific activities were found in tissues from a wide range of animal species including mammals [59-61]. Moreover, the ratio of these two activities varied depending on the tissue source [60,61]. Weitzman *et al.* [61] showed that these activities resulted from two distinct and separable enzymes, each enzyme having specificity for one of the two nucleotides. It has been suggested that the ATP- and GTP-specific enzymes might play distinct metabolic roles in animal tissues [17].

Succinyl-CoA synthetase activity is important for ketone body breakdown [17]. High concentrations of succinyl-CoA must be maintained for continued utilization of this pathway [20]. Since values of 100 have been estimated for the GTP / GDP ratio in mammalian mitochondria, a GTP-specific enzyme would be expected

to maintain high steady state levels of succinyl-CoA. Thus, GTP-specific succinyl-CoA synthetase should provide adequate substrate concentrations for CoA transferase activity in the ketone pathway [17]. In support of this proposal, Jenkins and Weitzman [62] demonstrated that the level of GTP-specific activity is significantly increased in tissues of ketotic animals while the levels of ATP-specific enzyme decreased. Since values closer to unity have been given for the ATP / ADP ratio, ATP-specific activity is more likely to be influenced by the levels of succinyl-CoA. Thus an ATP-specific enzyme is considered to be the more preferred form for operation within the TCA cycle. Studies in Trypanosomes have provided evidence to support this concept [63]. Trypanosomes also contain both ATP- and GTP-specific succinyl-CoA synthetases. TCA cycle activity is repressed when the organism is present in the mammalian bloodstream, whereas the procyclic form in the insect gut displays a fully functional cycle. Increases in the ATP-specific activity accompany the change to a procyclic form while the GTP-specific activity remains relatively low [63]. This is consistent with the role of the ATP-specific form in the TCA cycle.

It would appear that nucleotide specificity provides a means for metabolic compartmentalization of enzyme activity. As part of the TCA cycle, the ATP-specific enzyme operates in the direction of succinate synthesis driven by the continued production of succinyl-CoA in the oxidative cycle, while the GTP-specific enzyme operates in the reverse direction under high GTP / GDP ratios (Fig. 1.2). Further to its proposed role in ketone body utilization, a GTP-specific enzyme is likely involved in the anabolic pathways leading to heme and

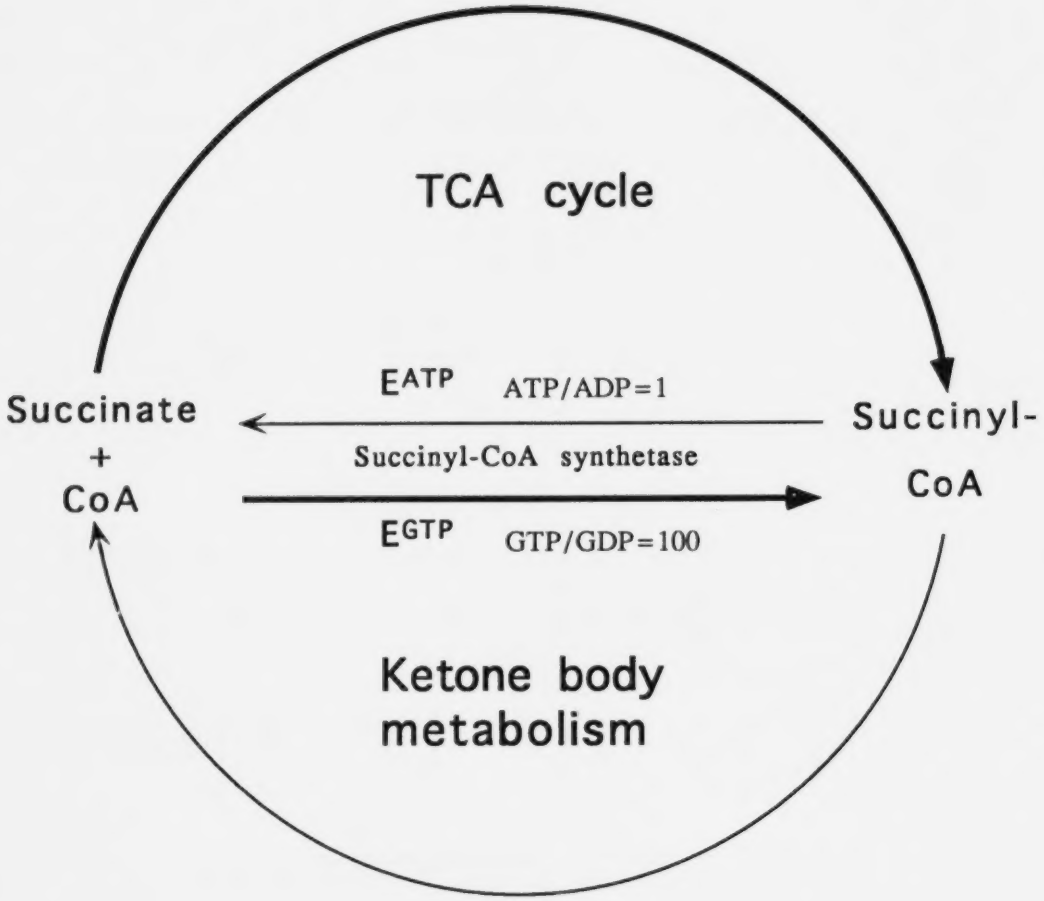

Figure 1.2. Metabolic compartmentalization of succinyl-CoA synthetase activity. As part of the TCA cycle, succinyl-CoA synthetase (E^{ATP}) converts succinyl-CoA to succinate and CoA, coupled to the production of ATP. Driven by the high GTP/GDP ratio, a GTP-specific (E^{GTP}) enzyme is proposed to play the role of maintaining a continuous supply of succinyl-CoA for ketone body breakdown [17].

cystathionine synthesis. These various reports are indicative of genetic polymorphism in eukaryotes. In this regard, it is worth noting that multiple genes of the α-subunit have been proposed in the yeast, *S. cerevisia* [64]. Knock-out mutations in a known α-subunit gene failed to produce the expected respiratory defects. In addition, heterologous sequences were detected from low stringency hybridization of yeast genomic DNA.

E. Biogenesis and assembly

In *E. coli*, the *suc*C gene encodes the β-subunit and the *suc*D gene encodes the α-subunit [6]. The termination codon of the *suc*C gene overlaps the initiation codon of the *suc*D gene by a single base pair. Similar overlapping is seen with the genes from other bacterial species [65,66]. In addition, a ribosome binding site is found several base pairs upstream of the overlap point [6,65,66]. It is thought that this arrangement serves to coordinate the production of the subunits in equimolar amounts for assembly. Neither of the subunits were found to accumulate to any extent in growing cells [67]. The two genes are cotranscribed with the subunits being translated from a single polycistronic message [68]. The subunits are produced and assembled in the cytosol of the cell. Folding and assembly has been shown to occur without the assistance of chaperonins [69]. It is worth noting that components of the α-ketoglutarate dehydrogenase complex are produced from the very same polycistronic message [70]. This adds significance to the finding that α-ketoglutarate dehydrogenase interacts specifically with succinyl-CoA synthetase

[71]. Out of seven enzymes tested, only succinyl-CoA synthetase was found to form an association with the dehydrogenase complex.

Assembly of the *E. coli* enzyme has been extensively studied *in vitro* [72-74]. Subunits that have been purified from the native enzyme under denaturing conditions will refold back into the active tetramer following removal of the denaturant [72]. Recovery of activity is dependent on protein concentrations in the refolding mixtures, decreasing markedly as the concentration falls below 25 μg/ml [73]. The refolding process is noticeably enhanced by the presence of either ATP or P_i [72,73]. This observation suggests that the occupation of the phosphoryl-binding site in the enzyme facilitates productive intrasubunit interactions. Recovery yields of up to 50 - 60 % can be achieved from such reconstitutions [72,73]. Khan and Nishimura [74] have shown that recovery of enzyme activity involves two sequential events. The first of these, the acquisition of secondary structure and association of the subunits into a tetrameric form is a rapid process being complete within minutes after renaturation. This is followed by a slower reactivation process involving subtle conformational rearrangements in the tetramer. It is this step in the renaturation process that is influenced by the presence of ATP. Greater than 90 % of the activity can be recovered from the denatured enzyme

In contrast to the bacterial system, assembly of the mammalian enzyme is less well characterized. Subunits of the mammalian enzyme are initially synthesized in the cytosol of the cell as higher molecular weight species called precursors. These precursors have N-terminally located signal sequences that serve to direct the

translocation of the subunits into the mitochondrial matrix [75,76]. The signal sequences are removed during the translocation process, releasing the mature subunits in the matrix where they assemble into the dimeric enzyme [77]. At present, no information is available on the assembly of this enzyme *in vivo*. Studies have, however, been conducted on the subunits of the native enzyme *in vitro*. In a manner similar to the bacterial case, reconstitution of the mammalian enzyme has been demonstrated following renaturation of a mixture of the isolated subunits [78]. The process of assembly is dependent on the presence of either glycerol or polyethylene glycol. This may be reflective of the unusually high protein concentrations found in the mitochondrial matrix. With the mammalian enzyme, GTP does not influence the assembly of the active dimer.

IV. THE MOLECULAR REACTION

A. THE REACTION MECHANISM

Succinyl-CoA synthetase catalyzes a number of partial reactions in which an exchange of isotope has been observed [79,80]. These findings suggested that the overall reaction proceeds through the formation of reaction intermediates. Table 1.II lists the various exchange reactions and their substrate requirements. Essentially each exchange is representative of one step in a sequence of reactions leading to net catalysis.

Kaufman [79] observed an exchange of isotope between ADP and ATP in the presence of Mg^{2+} alone. He interpreted the exchange in the absence of other reaction components to indicate the formation

Table 1.II

Exchange reactions of succinyl-CoA synthetase

Exchange Reaction	Substrate Requirements	Reference
ADP^{32} with ATP	Mg^{2+}	[39,79]
[^{18}O] phosphate with succinate	ATP and CoA or succinyl-CoA	[80]
[^{14}C] succinate with succinyl-CoA	P_i and Mg^{2+}	[39,79]

of a phosphorylated enzyme intermediate. Upper [81] confirmed this by demonstrating the covalent incorporation of label into the *E. coli* enzyme following incubation with [γ-^{32}P]ATP. Succinyl-CoA synthetase from pig heart was phosphorylated in a similar manner [82]. The enzyme is phosphorylated on a histidine residue [83,84]. Hultquist characterized the phosphorylated amino acid as the 3-phosphohistidine isomer [85]. The equilibrium constants for the formation of phosphoenzyme with NTP were estimated as K_{eq}= 31 for the *E. coli* enzyme [29] and K_{eq}= 2.1 for the mammalian enzyme [35]. The phosphorylated form of the enzyme is thus favored in both cases. This is consistent with the general observation from most laboratories that freshly prepared enzyme is found primarily in the phosphorylated form. Steady state kinetic measurements confirmed that the phosphoenzyme is an obligatory intermediate in catalysis of the overall reaction [86].

A second reaction intermediate, succinyl phosphate, was considered on the basis of [^{18}O] exchange data. Cohn [87] first observed the transfer of oxygen atoms of inorganic phosphate to the carboxyl oxygen of succinate during oxidation of α-ketoglutarate in mitochondria. This exchange was later shown to occur in the reaction catalyzed by succinyl-CoA synthetase [80,88]. Succinyl phosphate was proposed as an intermediate through which direct transfer could take place. Nishimura and Meister [89] demonstrated the formation of succinyl [^{32}P]phosphate from reaction mixtures of succinate, [γ-^{32}P]ATP and substrate quantities of enzyme. Furthermore synthetic succinyl phosphate phosphorylates the enzyme and reacts with ADP producing ATP and with CoA producing succinyl-CoA. Succinyl

phosphate is not released from the enzyme during net catalysis. Measurements of the rate constants suggest succinyl phosphate reacts with CoA faster than it dissociates from the enzyme [49]. Under the appropriate conditions, succinyl phosphate reacts nonenzymatically with CoA to form succinyl-CoA [50]. The rate of this nonenzymatic reaction approximates that measured for the partial reaction with the enzyme. This suggests that the final step in the formation of succinyl-CoA may proceed in a nonenzymatic fashion on the surface of the protein. A mechanism for the nonenzymatic reaction has been proposed [90]. In this proposal, the free carboxyl group interacts with the carboxyl phosphate group forming a cyclic intermediate. This then allows for an attack by the thiol group of CoA (Fig. 1.3).

Combining all of the above data leads to the proposal that the enzymatic reaction proceeds through three sequential steps described by the following set of equations:

$$E + NTP \overset{Mg^{2+}}{\rightleftharpoons} E\text{-}P + NDP$$

$$E\text{-}P + \text{succinate} \rightleftharpoons E.\text{succinyl phosphate}$$

$$E.\text{succinyl phosphate} + CoA \rightleftharpoons E + \text{succinyl-CoA} + P_i$$

B. Kinetics and binding constants

Steady state kinetics have been carried out on both the pig heart [91] and the *E. coli* [92,93] enzymes. With the pig heart enzyme, GTP and CoA are the first substrates to associate with the enzyme. They add in a random order. The kinetics show that the third substrate, succinate enters the pathway either before or after

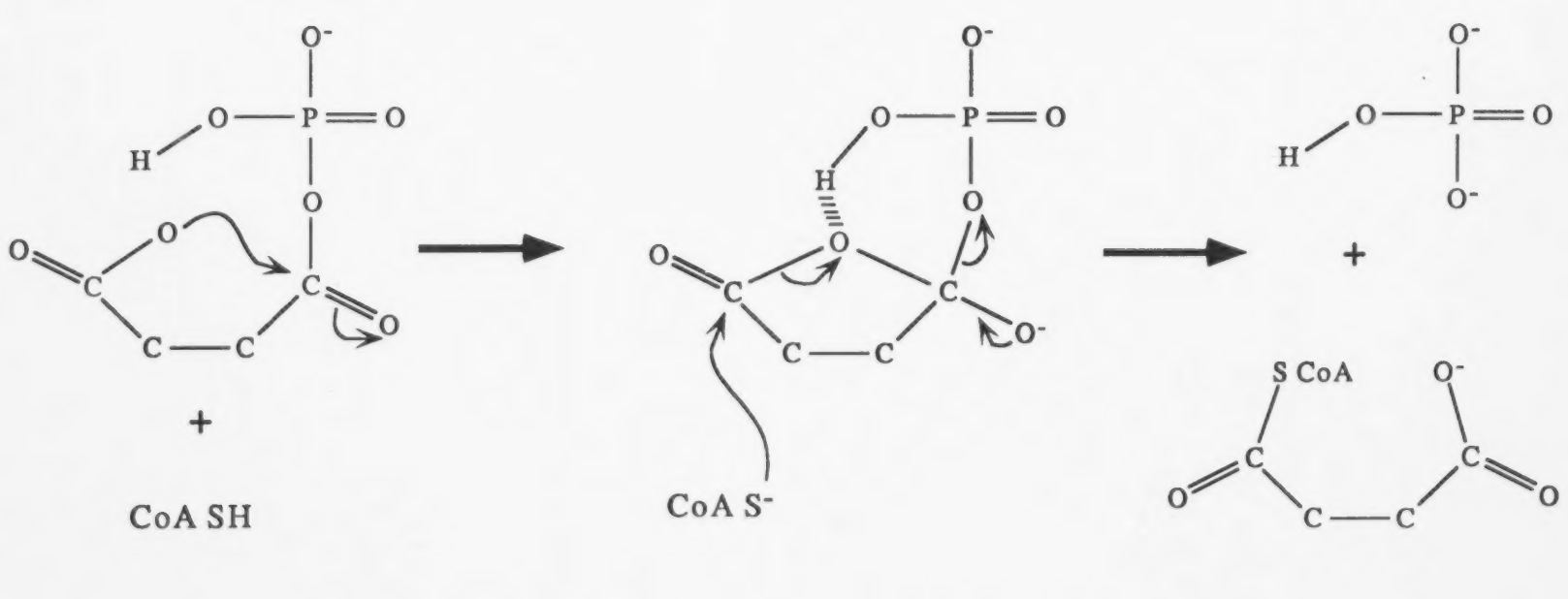

Figure 1.3. The nonenzymatic reaction of succinyl-phosphate with CoA. The reaction scheme illustrated above was proposed by Walsh *et al.*, 1970 [90] to account for the nonenzymatic reaction of chemically synthesized succinyl-phosphate with CoA to produce succinyl-CoA and inorganic phosphate.

the product, phosphate leaves. Succinyl-CoA and GDP are last to dissociate from the enzyme and do so in a random order (Fig. 1.4*a*). The formation of succinyl phosphate as an intermediate depends on the entry of succinate before phosphate leaves the pathway. If phosphate were to leave before succinate added to the enzyme then abortive complexes would be formed and net catalysis would not take place [91]. The kinetics of the *E. coli* enzyme show a less ambiguous sequence of substrate addition and product release [93,94]. The mechanism that has been proposed for this form of the enzyme is described as partially random fully sequential. ATP is the first substrate to enter while ADP is last to leave the pathway. Entry of the remaining substrates and release of the other two products occur in a random order according to the scheme outlined in Figure 1.4*b*.

The Michaelis constants of the pig heart [91], *E. coli* [92,93] and artichoke [41] enzymes for the various substrates have been determined. Table 1.III lists the k_m values for the three enzymes. k_m values for ADP and ATP and for CoA and succinyl-CoA are one to two orders of magnitude lower than those for succinate and phosphate. These differences are reflected in the general order of substrate addition and product release during catalysis. Those substrates with lower k_m values, namely the nucleotides, tend to add to the enzyme first and dissociate last.

C. Substrate Synergism and Conformational Changes

The rate of the partial exchange reaction between ATP and ADP is significantly slower than the overall reaction rate [29]. The rate of

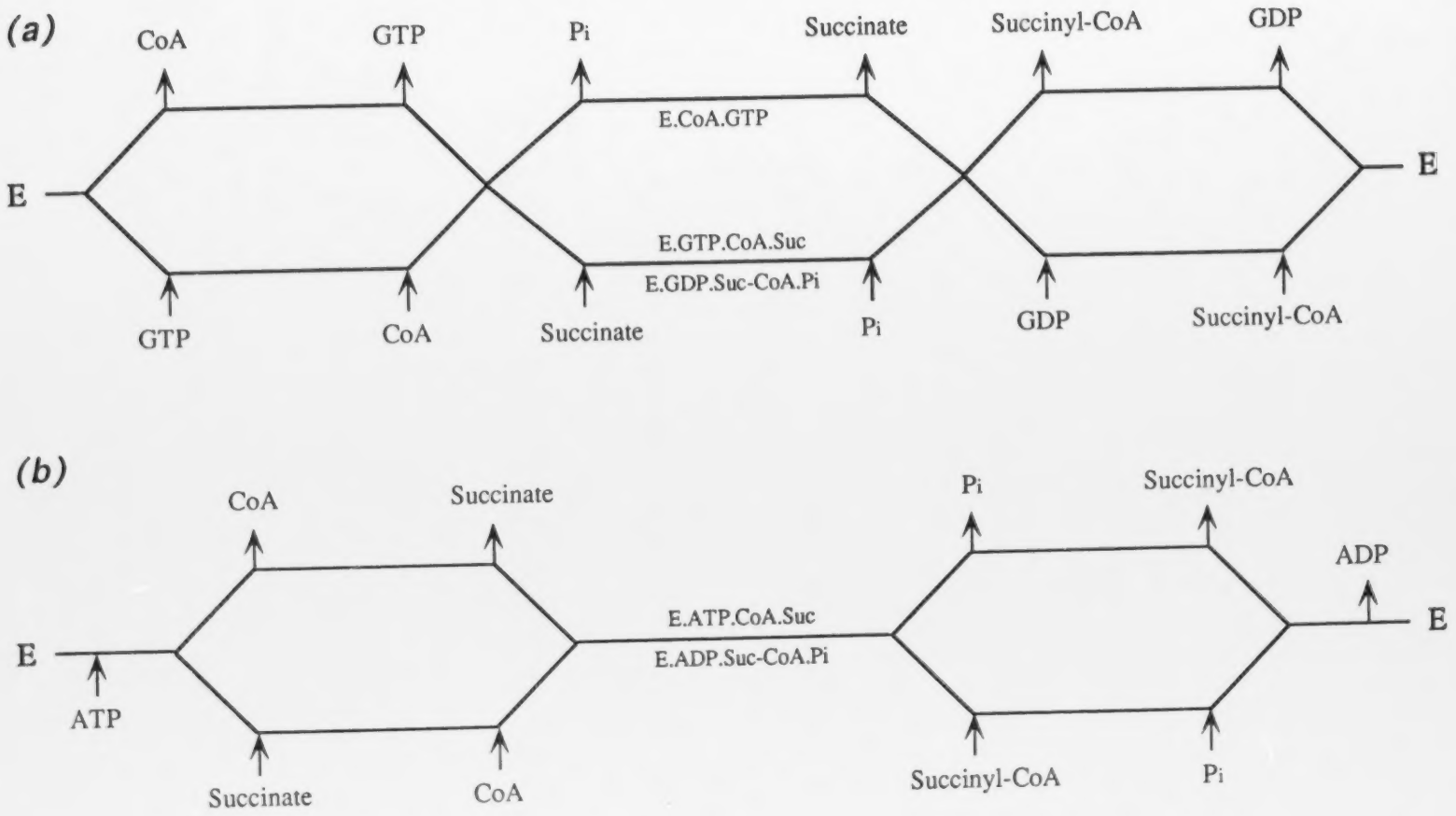

Figure 1.4. Reaction schemes for succinyl-CoA synthetase from *E. coli* and pig heart. The orders with which the substrates and products associate and dissociate from pig heart succinyl-CoA synthetase are shown in ***(a)*** and from *E. coli* succinyl-CoA synthetase in ***(b)***. The sequences of addition and release were deduced from steady state kinetic measurements [91-93]. The productive catalytic pathway of the enzyme (**E**) proceeds through the formation of the ternary complexes E.NTP.CoA.Suc and E.NDP.Pi.Suc-CoA.

Table 1.III

Kinetic constants of succinyl-CoA synthetase

Substrate	***E. coli*** [92,93]	**Pig heart** [91]	**Artichoke** [41]
Succinyl-CoA	7.7×10^{-6}	3.0×10^{-5}	5.6×10^{-5}
CoA	1.5×10^{-6}	2.0×10^{-5}	2.2×10^{-6}
ADP / GDP *	1.2×10^{-5}	8.0×10^{-6}	1.2×10^{-4}
ATP / GTP *	2.0×10^{-5}	1.0×10^{-5}	1.4×10^{-4}
Succinate	1.0×10^{-4}	8.0×10^{-4}	2.0×10^{-3}
P_i	2.6×10^{-3}	6.0×10^{-4}	1.4×10^{-3}

* ADP and ATP were used as substrates for the *E. coli* and artichoke enzymes, GDP and GTP for the pig heart enzyme.

exchange is stimulated by the presence of succinyl-CoA [29,86]. This effect has been described as "substrate synergism" wherein the enzyme is not fully active until all the binding sites are occupied by substrates. Synergistic effects are also seen with substrate analogues such as desulfo-CoA. This analogue dramatically stimulates the formation of succinyl phosphate from ATP and succinate [94] and the synthesis ATP from ADP and succinyl phosphate [50]. The fact that synergism is seen in the dimeric pig heart enzyme clearly shows that it operates within a single active site [95]. Synergism may be a manifestation of conformational changes that occur in the enzyme following the binding of substrates.

Phosphorylation is known to cause a change in the conformation of the enzyme. The dephosphorylated form of the *E. coli* enzyme loses activity on storage more rapidly than the phosphoenzyme. The rate of inactivation by trypsin was two orders of magnitude greater for the dephosphorylated form [96]. The phosphoenzyme was, in fact, almost completely protected from proteolytic inactivation. This would seem to indicate a change to a more compact conformation following phosphorylation of the enzyme. Binding of either CoA or ATP to the *E. coli* enzyme causes an enhanced quenching of tryptophan fluorescence by acrylamide [97]. Furthermore, CoA binding induced changes in the flexibility of the protein in regions labeled with dansyl chloride [98]. These observations are consistent with alterations in conformation.

Proton relaxation rate (PRR) studies using Mn(II) as a probe show that significant structural changes occur at the active site of the enzyme [99]. The binding of CoA to the E.Mn and E-P.Mn complexes

produced increases in the PRR enhancement. Addition of both CoA and succinate together caused a large decrease in enhancement, whereas succinyl-CoA produced an increase in the enhancement. Thus, binding of different substrates have noticeably different effects on the conformation of the active site. Similar structural rearrangements were detected in ^{31}P-NMR studies [100]. The addition of CoA to E-P caused a downfield shift and a broadening of the phosphohistidine resonance. The broadening was interpreted as being indicative of two exchanging conformations, one allowing for phosphoryl transfer to ADP, the other for transfer to succinate. When CoA and the competitive inhibitor, 2,2'-difluorosuccinate were added the resonance narrowed suggesting that the phosphoryl group was frozen in one of these orientations.

A significant finding from the PRR, NMR and fluorescence studies was that the addition of succinate or its inhibitor alone produced little change in the conformation of the protein [97,99,100]. Thus, the major changes in conformation occur following binding of CoA and phosphorylation by ATP. These observations suggest a model in which the enzyme is "charged" by phosphorylation and binding of CoA. The subsequent binding of succinate acts like a "trigger" firing the reaction. In this way, the enzyme ensures that the entire reaction is carried out. Thus, conformational changes required for catalysis provide an elegant mechanism for coupling the three steps in the reaction sequence. The consequences of these conformational changes are schematically illustrated in Figure 1.5. The model is consistent with the reaction pathway followed by the pig heart enzyme.

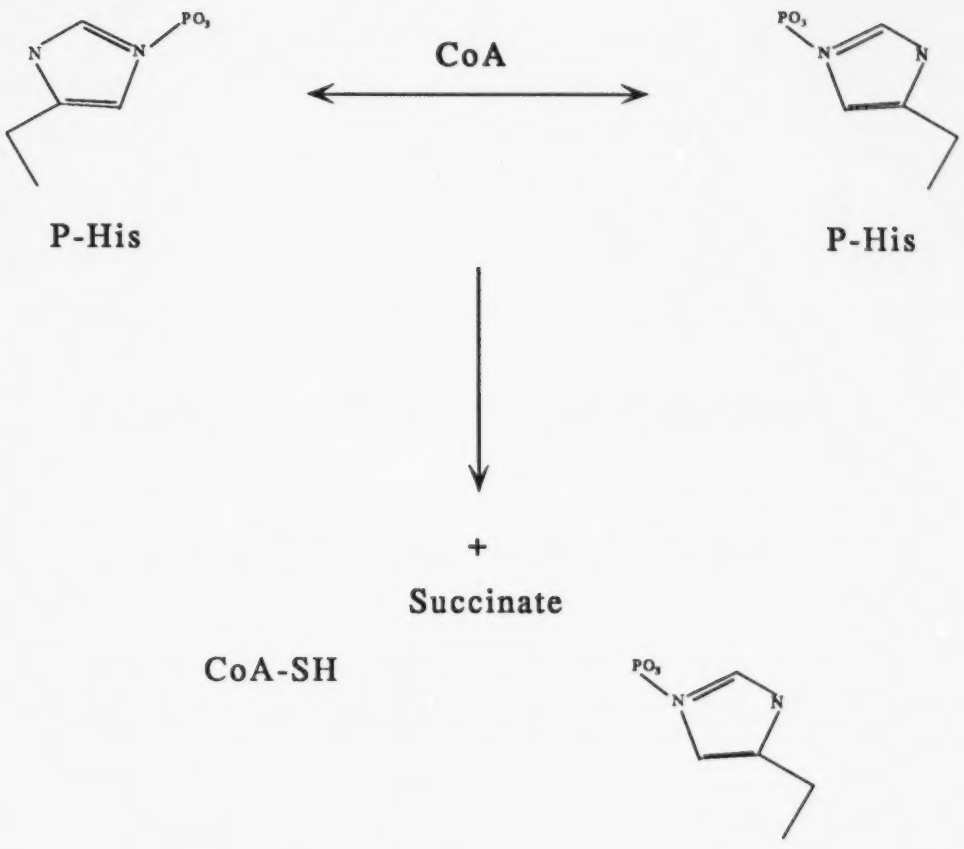

Figure 1.5. Conformational rearrangements in the phosphorylated enzyme. Shown above is a diagramatic representation of the structural changes that are proposed to occur at the active site of succinyl-CoA synthetase. The phosphohistidine (P-His) exists in two conformations in the presence of CoA. Addition of succinate to the enzyme locks the phosphohistidine residue into one of these conformations to acheive net catalysis.

D. ALTERNATING SITES COOPERATIVITY

Several early studies suggested that the *E. coli* enzyme incorporated just one mole of phosphate per mole of enzyme [24,29,96]. Since there are two possible active sites in the tetramer, the results were interpreted as evidence for a "half of sites" reactivity in the enzyme [96]. However, stoichiometries closer to two moles of phosphate per mole of enzyme were achieved in some studies [29,94]. The lower values, it was argued, could be attributed to the use of enzyme preparations with low specific activities [101].

Nevertheless, further evidence was sought to support the "half of sites" model. Bild *et al.* [102] tested the enzyme for catalytic cooperativity by measuring the extent of oxygen exchange between medium [^{18}O]phosphate and succinate per molecule of ATP cleaved under steady state conditions. An increase in oxygen exchange was detected as ATP concentration were lowered. The dimeric pig heart enzyme showed no change in oxygen exchange. An alternating sites cooperativity was proposed to explain their observations. In the model, the binding of ATP at one site facilitates the formation and release of succinyl-CoA at the other site. Subsequent phosphorylation at the first site would then prepare this site for the next catalytic turnover. Wolodko *et al.* [103] found that the active sites of the *E. coli* enzyme have the capacity for alternating activity. Furthermore, the rate of discharge of thiophosphate from the enzyme in the presence of succinate and CoA is stimulated by ATP as would be predicted by the model [104]. Reaction of the thiophosphorylated enzyme with ADP was also stimulated by succinyl-CoA [105]. Nonhydrolyzable ATP analogues were without stimulatory effect

[104]. However, thiophosphate discharge did not appear to be accompanied by phosphorylation at the second site as was suggested to occur in the model [105].

Although corroborating evidence for the alternating sites model was provided from a ^{31}P-NMR study of the enzyme [100,106], it was pointed out that the pig heart enzyme at high concentrations exhibits properties similar to those seen with the *E. coli* enzyme [106]. Following this report, Nishimura and Mitchell [107] demonstrated that dephosphorylation of the thiophosphorylated pig heart enzyme was also stimulated by ATP. Thus, in cases where results was used as evidence in favor of cooperativity for the two site tetramer, the single site dimeric enzyme was found to display the same characteristics. The data was reinterpreted as a simple displacement mechanism at a single site exhibiting substrate synergism [4,107]. With the availability of the genes for the *E. coli* enzyme, Mann *et al.* [107] were able to create a hybrid tetramer in which one of the active sites was inactivated by mutation of the catalytic histidine residue. The hybrid was catalytically active and displayed the properties typical of substrate synergism. Thus, it was concluded that the tetrameric *E. coli* enzyme is comprised of two independently active dimers associated to form a "dimer of dimers".

V. STRUCTURE / FUNCTION RELATIONSHIPS

A. PRIMARY STRUCTURE OF THE SUBUNITS

The *E. coli* enzyme was the first succinyl-CoA synthetase to be genetically identified and characterized [6]. The genes are located at

one end of a gene cluster that encodes several TCA cycle enzymes. The *suc*C gene encodes a 388 amino acid β-subunit while the *suc*D gene encodes a 288 amino acid α-subunit. The calculated molecular masses, 29.6 kDa (α) and 41.4 kDa (β), were consistent with the previously estimated values [28,29]. Since the discovery of the *E. coli* genes, sequences encoding one or both subunits of the enzyme from six other sources have been reported [64-66,75,76,109-111]. The amino acid sequences of the α-subunits have been aligned and are shown in Figure 1.6. An alignment of the presently available β-subunits can be found in Bailey *et al.* [76]. Both subunits exhibit a high level of conservation. Of the two, the α-subunit shows the higher degree of identity. However, within the β-subunit sequences, the C-terminal one third of the protein displays a higher level of conservation than the N-terminal two thirds [76]. An explanation for this became apparent following analysis of the crystal structure [SECTION V. B]. The mammalian subunits are slightly larger than those of the bacterial enzyme. The α-subunit is calculated to be 32.1 kDa [75], while the β-subunit is 42.5 kDa [76]. When the sequences of the mammalian and *E. coli* subunits are compared, the α-subunits show 70 % identity, whereas the β-subunits are just 45 % identical.

The catalytic histidine that is phosphorylated during the course of the reaction is found in the α-subunit [47]. The phosphorylated *E. coli* enzyme was digested with trypsin. A labeled peptide fragment was isolated from the digest and its amino acid sequence was determined [112]. The sequence of this peptide matched residues 244-255 of the *E. coli* α-subunit and identified αHis_{246} as the catalytic histidine [6]. This histidine is conserved in all the

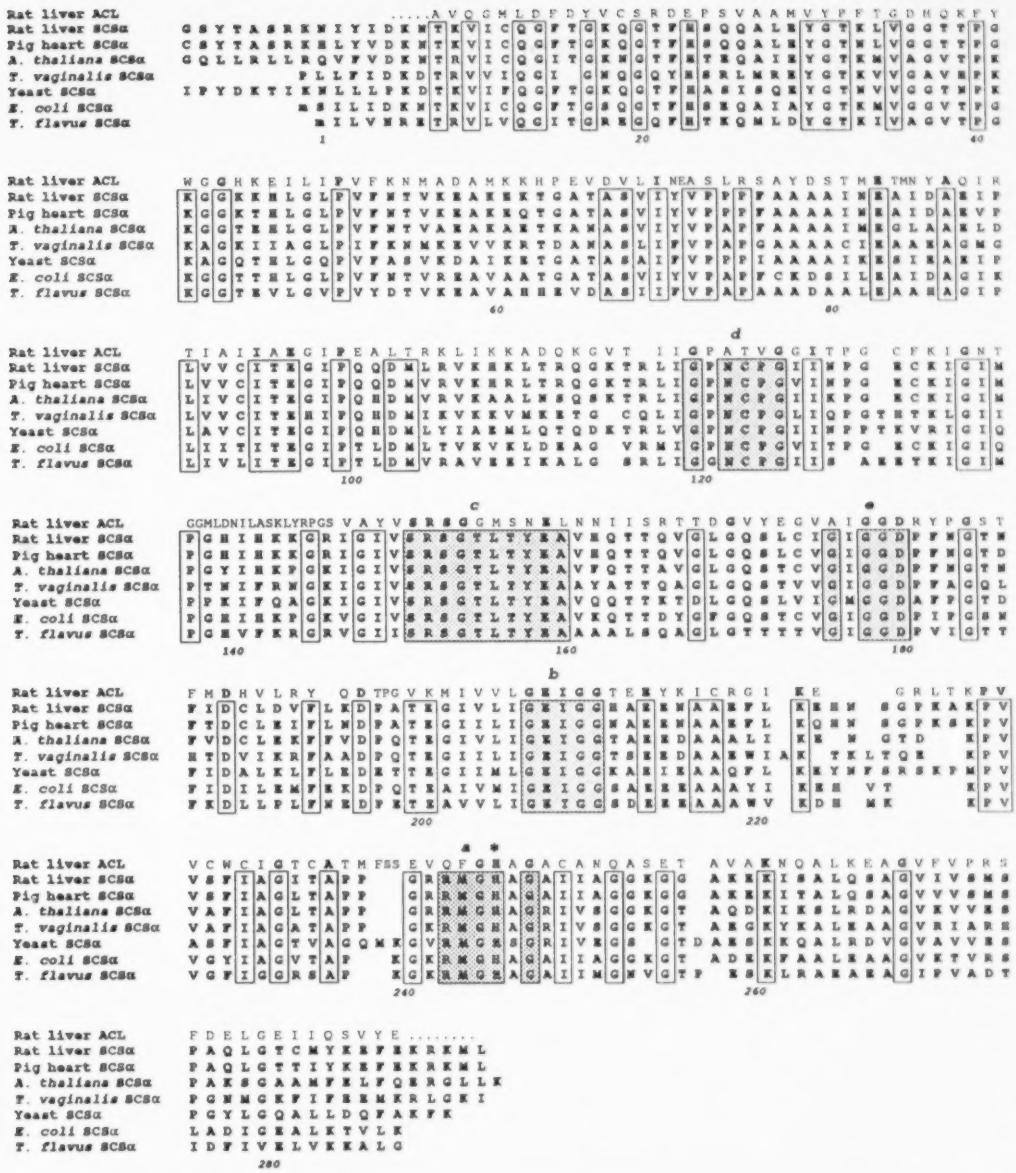

Figure 1.6. Comparison of the amino acid sequences of the α-subunits of succinyl-CoA synthetase (SCS). The sequences of the various subunits are taken from the following: Rat ATP citrate lyase, [114]; Rat SCS, [75]; Pig SCS, [Chap. 4]; *Arabidopsis thaliana*, [Swiss Prot. Data base]; *Trichomonas vaginalis*, [109]; Yeast, [64]; *E. coli*, [6]; and *Thermus flavus*, [66]. Boxes indicate positions of identity among the α-subunit sequences. Shaded boxes highlight stretches of conseved sequence and are identified by a letter (*a-e*). Asterix denotes the position of $His_{246}\alpha$. The numbering below the alignment is from the *E. coli* sequence.

α-subunits. In addition residues around the histidine in the primary sequence are highly conserved (Fig. 1.6*a*). Replacement of this histidine residue by either aspartate [7] or asparagine [108] results in complete loss of catalytic activity in the *E. coli* enzyme. Furthermore, the $\alpha His_{246}Asp$ mutant fails to catalyze a partial reaction that does not involve the participation of the phosphohistidine intermediate [113]. This indicates that the presence of the histidine is required to form the correct microenvironment within the active site over and above its role in forming an intermediate.

The primary structure of rat liver ATP citrate lyase was determined recently [114]. The enzyme is responsible for the production of acetyl-CoA for lipogenesis in eukaryotes [18]. The enzyme catalyzes the formation of acetyl-CoA and oxaloacetate from citrate and CoA through the intermediate formation of citryl-CoA. The reaction is accompanied by hydrolysis of ATP to ADP [115]. The mechanism bears striking similarity to that of succinyl-CoA synthetase including the formation of a phosphohistidine intermediate. It was not surprising, then, to find that part of the primary sequence of ATP citrate lyase shows significant similarity to the α-subunit of succinyl-CoA synthetase [114]. Many of the residues found to be identical among the α-subunits are also conserved in ATP citrate lyase (Fig. 1.6). Moreover, the catalytic histidine is found in an equivalent position within the aligned sequence of ATP citrate lyase.

B. THE CRYSTAL STRUCTURE

The x-ray crystal structure of succinyl-CoA synthetase from *E. coli* was determined at 2.5 Å resolution [8]. In the structure, the α-subunits lie at opposite poles of the tetramer, each interacting primarily with one of the β-subunits (Fig. 1.7). The β-subunits, in addition to their interactions with the α-subunits, associate with each other to form the dimer of αβ-dimers. The role of the β-subunit in the formation of the tetramer was previously suggested from cross-linking studies [116]. The β-subunits associate through several salt bridge and H-bonding interactions. Residues involved in these interactions are found within the N-terminal two thirds of the subunit. This explains in part why the N-terminal part of the β-subunit displays a lower sequence conservation than the C-terminus [76]. In particular, those residues of the *E. coli* β-subunit that contribute to the tetramerization are not conserved in the β-subunit from the dimeric mammalian enzyme. One or both members of the various pairs of interacting residues are changed in the eukaryotic enzyme [8].

Both α- and β-subunits are folded into distinct N-terminal and C-terminal domains. As already mentioned, the N-terminal domain of the β-subunit is involved in forming the dimer of αβ-dimers. The two domains of the α-subunit and the C-terminal domain of the β-subunit provide much of the known structural elements of the active site. Each of these domains contain a nucleotide-binding motif described as a "Rossmann fold" [117]. In the structure, CoA is bound to the N-terminal domain of each of the α-subunits (Fig. 1.8*a*). A short helix from the N-terminal domain of the β-subunit reaches over and forms

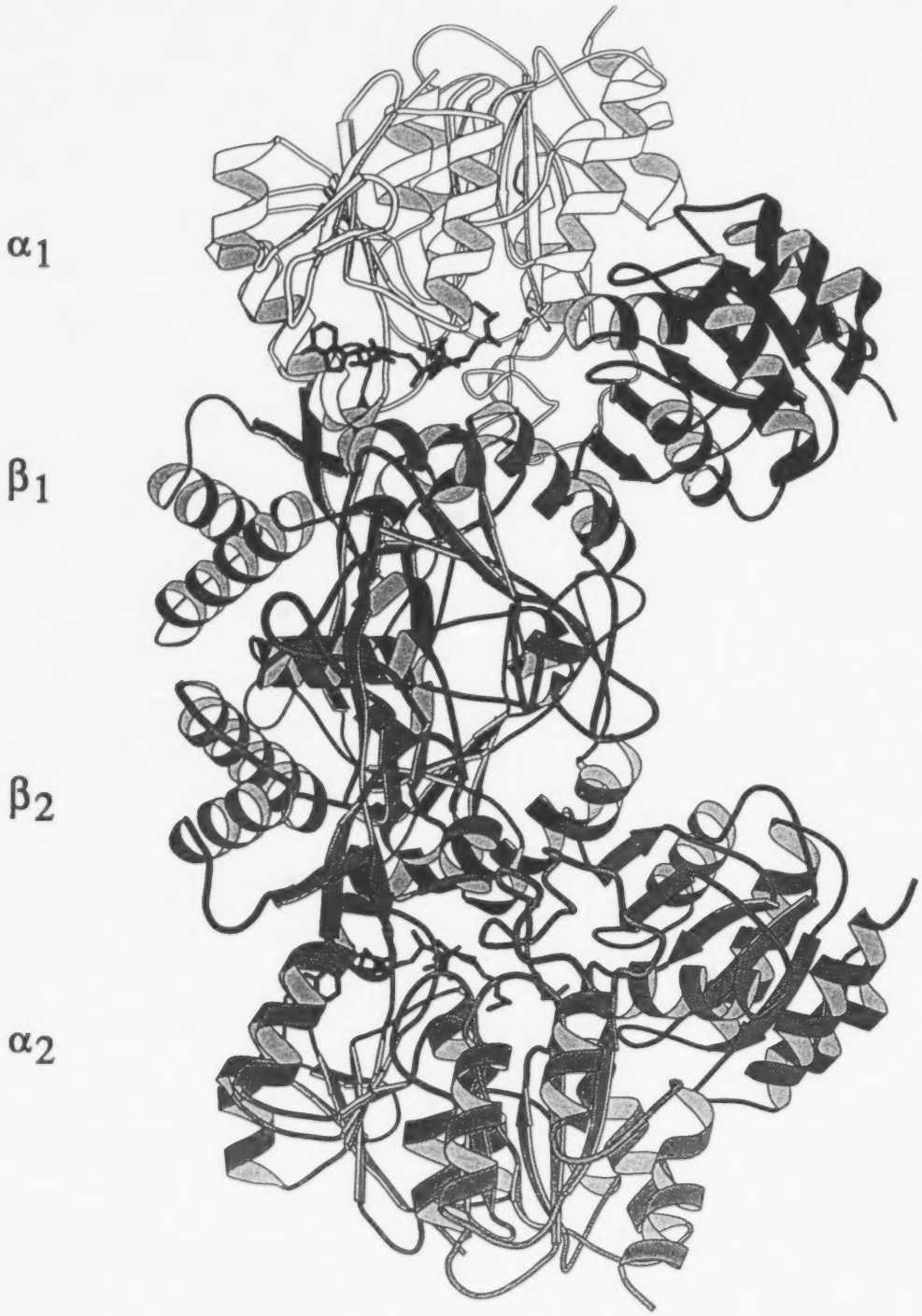

Figure 1.7. Schematic representation of the tetrmer of succinyl-CoA synthetase from *E. coli*. The non-crystallographic 2-fold axis of symmetry relating the $\alpha_1\beta_1$-dimer to the $\alpha_2\beta_2$-dimer lies perpendicular to the page. This graphic was taken from Wolodko *et al.*, 1994 [8].

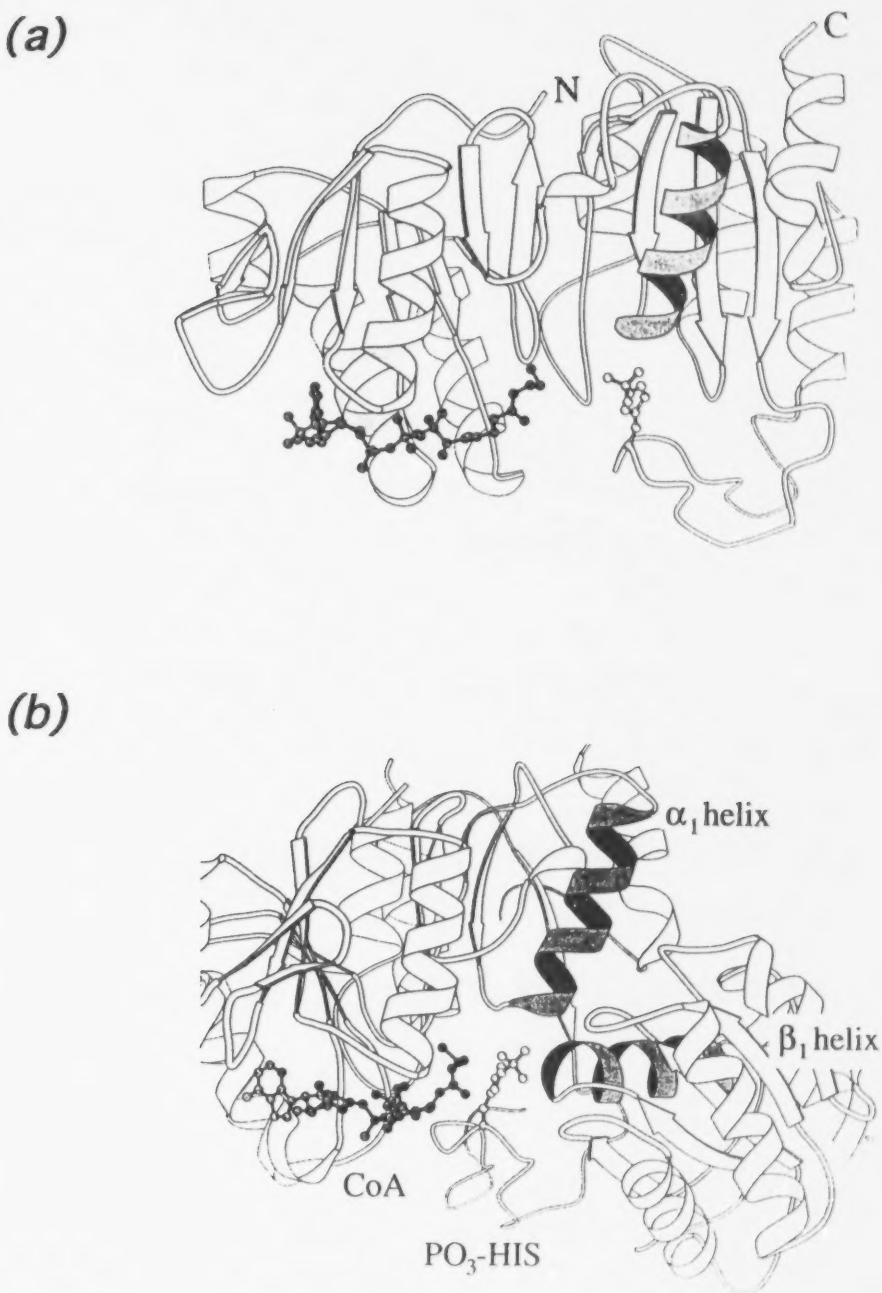

Figure 1.8. Schematic representation of the α- and β-subunits of the enzyme. The phosphorylated α-subunit is shown in ***(a)*** with a molecule of CoA bound to its N-terminal domain. Panel ***(b)*** shows the arrangement of two α-helices (shaded darker) at the active site pocket. These graphics were taken from Wolodko *et al.*, 1994 [8].

part of the CoA-binding site of the other αβ-dimer. Participation of a second β-subunit in the binding of CoA provides a rationale for the tetrameric structure of the *E. coli* enzyme. Furthermore, the presence of additional structural elements in the CoA-binding site of the bacterial enzyme may explain its higher affinity for CoA. The K_m of the *E. coli* enzyme for CoA is tenfold lower than that of the mammalian enzyme [91,92].

The catalytic histidine residue, αHis_{246}, is located on an extended loop in the C-terminal domain of the α-subunit (Fig. 1.8*a*). In the structure, αHis_{246} is phosphorylated in both of the α-subunits. This finding is significant to the earlier arguments over "half of sites" reactivity [96]. The bisphosphorylation of the enzyme together with the occupation of both CoA-binding sites argues strongly against an alternating sites cooperativity mechanism [102]. Clearly both active sites are similarly poised in their catalytic cycles.

Two of the oxygens of the phosphoryl group of phosphohistidine are situated close to the N-termini of α-helices and are coincident with the helix axis in both cases(Fig. 1.8*b*). One of these helices is part of the C-terminal domain of the α-subunit while the other is provided by the C-terminal domain of the β-subunit. The amino acid sequences of both of these helices are extremely well conserved. The helix that is part of the C-terminal domain of the α-subunit is comprised of the longest stretch of conserved residues in the protein (Fig. 1.6*c*). It is believed that the partial positive charges at the N-termini of these helices (resulting from helix dipoles) serve to stabilize the phosphohistidine residue [8]. This explains the unusually stable nature of the phosphoenzyme form. Two of the

phosphoryl oxygens are within hydrogen-bonding distance of residues at the N-termini of the helices. The side chain hydroxyl groups of αSer_{153} and αThr_{155} and the amide nitrogen of αGly_{154} interact with one of the oxygen atoms. Backbone amide nitrogen atoms of βAla_{266} and βGly_{267} hydrogen-bond with the second oxygen atom.

The carboxylate group of αGlu_{208} in the C-terminal domain of the α-subunit interacts with the imidazole ring of phosphohistidine at the N-1 position. αGlu_{208} is located on a loop connecting two secondary structures. The amino acid sequence of this loop is identical not only in the α-subunits but also in the aligned ATP citrate lyase (Fig. 1.6*b*). Phosphorylation of the histidine residue is envisioned as a nucleophilic attack by the N-3 atom on the terminal phosphoryl of ATP or on the phosphoryl of succinyl phosphate. Since the tautomeric form of the imidazole ring containing the lone pair of electrons on the N-1 is favored, it has been suggested that the tautomer with the lone pair at the N-3 is stabilized by an anionic group [100]. In the structure, αGlu_{208} is ideally positioned to play this role (Fig. 1.9).

The binding sites for succinate and ADP have still to be determined. There are two unoccupied nucleotide-binding motifs, one in each of the C-terminal domains of the α- and β-subunits. These two domains interact closely with one another and are both ideally positioned with respect to the phosphohistidine to provide the binding domain for ADP. The α-subunit alone was previously shown to be capable of phosphorylating its own histidine residue [118]. This suggests that the ADP-binding site is part of the

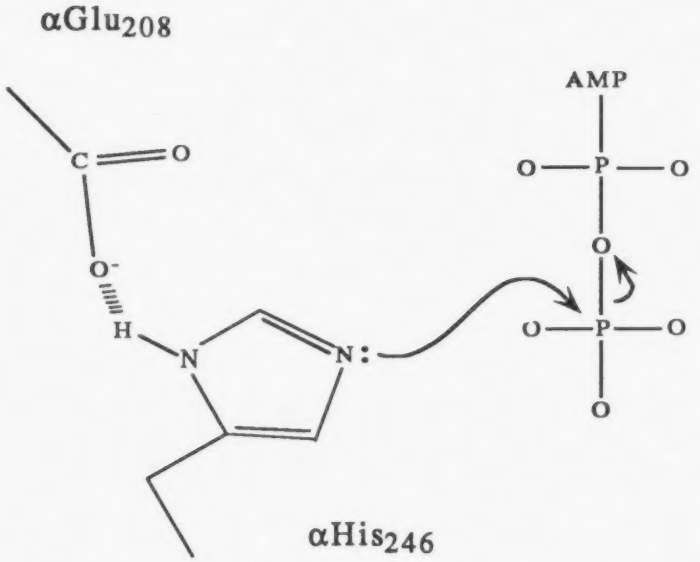

Figure 1.9. The role of αGlu_{208} in the phosphorylation of αHis_{246}. Shown above is a diagramatic representation of the positions of αGlu_{208} and αHis_{246} at the active site of succinyl-CoA synthetase. The carboxylate group of the glutamate residue is proposed to stabilize the tautomeric form of the imidazole ring of histidine in which the lone pair of electrons is on the N-3 nitrogen atom. This allows for an on-line attack by the N-3 nitrogen on the terminal phosphoryl group of ATP.

α-subunit. However, the catalytic rate of phosphorylation is several orders of magnitude slower in the absence of the β-subunit. Binding of the affinity label, ADP-dialdehyde resulted in cross-linking of the α- and β-subunits [119]. It is, therefore, a possibility that the binding site for ADP is shared between the two subunits. With regard to succinate, the thiol group of CoA extends to within 7 Å of the phosphoryl group of phosphohistidine. The oxygen atom not taken up by interactions with the "stabilizing" helices points directly toward the thiol group. Succinate could bind between these two groups with one carboxylate placed to react with the phosphohistidine and the other positioned to react with the thiol group. Interestingly, two highly conserved loops from the α-subunit (Fig. 1.6*d* & *e*) converge on this region and might contribute residues for binding of succinate.

VI. RESEARCH AIMS

A. Biogenesis of the Mammalian Enzyme

Subunits of mammalian succinyl-CoA synthetase must be imported into mitochondria prior to assembly of the enzyme. The subunits are synthesized in the cytosol of the cell as larger precursors. These precursors contain N-terminal signal sequences that direct the translocation of their attached subunits into the mitochondrial matrix. The signal sequences are removed during the translocation process releasing the mature subunits for assembly. Gaining insight into how this translocation is achieved is of central importance to understanding biological compartmentalization of cellular functions in eukaryotes.

It is generally accepted that polypeptides requiring translocation across biological membranes must be presented to the translocation machinery in a loosely folded conformation. The universal observation that signal sequences are removed from most translocated proteins suggests that their removal is a necessary prerequisite for folding and assembly to occur. Thus, it is possible that signal sequences may directly modulate the folding properties of their attached proteins to allow for translocation. The precursor form of the α-subunit can readily be assessed for this role. The mature subunit is capable of catalyzing its own phosphorylation. This indicates that the subunit must be able to form a conformation resembling that which is present in the native enzyme. Thus, the ability to autophosphorylate represents a sensitive assay for the conformation within the precursor. In addition, the precursor can be examined for its ability to assemble with mature β-subunit and reconstitute enzyme activity.

B. Isoforms of the Mammalian Enzyme

The polymorphic nature of the mammalian enzyme precluded this form of the enzyme from originally being selected for crystallographic studies. However, there are several reasons why this form of the enzyme would be a more desirable model for structural and mechanistic work. The mammalian enzyme is a dimer. Its smaller size should conceivably make diffraction data handle easier. Since the enzyme does not posses cooperativity, it should provide a better model on which to carry out mechanistic studies. The genetic identification of isoforms would further our understanding of the

relationship between polymorphism and metabolism. The enzyme from pig hearts traditionally been more difficult to purify from source than its bacterial counterpart, and yields of the final product are low. Isolating the genes would permit their expression in a bacterial host. In this way, homogenous preparations of the enzyme could be obtained and structural studies could proceed. Obtaining structural information for the dimer is of interest in understanding of changes that have occurred in the quaternary structure of the enzyme.

C. MUTAGENESIS OF THE *E. coli* ENZYME

Mutagenesis studies have been considered since the *E. coli* genes became available. Expression of the genes in a bacterial system was optimized making follow-up structural work feasibly. However, with only one primary sequence to examine and no crystal structure, only a hit and miss approach could be made. Consequently, many of the early mutations that were performed on the protein resulted in little alteration of catalytic activity. As more and more sequences were reported, comparisons were made and gradually strong sequence identity began to appear. This was especially evident in the α-subunit. With the structural information at hand by 1993, a more directed approach could be taken at dissecting the molecular reaction. Knowledge of the crystal structure together with the ability to carry out mutagenesis on the enzyme will provide a very good framework for future exploration.

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Section II

Biogenesis and Assembly

Chapter 2

Conditions for the production, purification and refolding of subunits of the *E. coli* enzyme

I. INTRODUCTION

Succinyl-CoA synthetase (SCS) provides an excellent model for investigating the process of folding and assembly of a protein with a heterologous subunit structure. The enzyme is comprised of α- and β-subunits of molecular masses ~30-32 kDa and ~42 kDa, respectively [1]. In *E. coli*, these subunits associate to form a tetramer of the type $\alpha_2\beta_2$. Assembly of the *E. coli* enzyme has been well studied *in vitro* [2-4].

Pearson and Bridger [2] demonstrated that the enzyme can be reconstituted from mixtures of subunits that had been previously separated from the native enzyme under denaturing conditions. Recovery of enzyme activity is dependent on the protein concentrations in the refolding mixtures, decreasing markedly as the concentration falls below 25 μg/ml [3]. The presence of either of the substrates, ATP or P_i, enhanced the recovery of activity [2,3]. This observation suggests that occupation of the phosphoryl-binding site in the enzyme facilitates productive intrasubunit interactions. Under optimal conditions, recovery yields of 50-60 % can be achieved from such reconstitutions [2,3]. Studies on the renaturation of the *E. coli* enzyme have shown that recovery of activity involves two sequential events [4]. The first of these, the acquisition of secondary structure and association of the subunits into the tetramer, occurs within minutes after renaturation. This is followed by a slower reactivation process involving subtle conformational rearrangements. It is this second step that is influenced by the presence of ATP [4].

The genes for both subunits of the *E. coli* enzyme have been isolated and characterized [5]. The *suc*C gene encodes the β-subunit while *suc*D encodes the α-subunit. The two subunits are produced from a single polycistronic transcript [6]. The termination codon of *suc*C overlaps the initiation codon of *suc*D by a single base pair. Furthermore, a ribosome binding site is found several base pairs upstream from the overlapping residue, within the 3' end of the *suc*C gene [5]. It is thought that this arrangement serves to coordinate the synthesis of the two subunits in equimolar amounts for assembly. This is supported by the finding that neither of the subunits were found to accumulate to any extent in growing cells [7]. A similar overlapping is seen with the genes from other bacterial species [8,9]. Assembly of the bacterial enzyme takes place in the cytosol of the cell and has been shown to occur in the absence of the chaperonin, GroEL [10].

The original reconstitution experiments were performed with subunits that had been purified from the native enzyme by gel filtration under denaturing conditions. Preparing subunits in this manner is both time consuming and labor intensive. The *E. coli* enzyme can be efficiently overproduced using bacterial expression systems [11]. Rapid isolation of the subunits could be achieved if the two genes were separated and expressed as individual units. Such a recombinant-DNA based method for producing the subunits would provide a more versatile system in which to carry out further folding and assembly studies.

II. MATERIALS AND METHODS

A. BACTERIAL STRAINS, PLASMIDS AND REAGENTS

The following strains of *E. coli* were used in these studies: JM109 was employed for molecular cloning; uracil-enriched DNA used for mutagenesis was generated in CJ236; expression was carried out in TK3D18, a strain in which the SCS genes have been deleted. The genotypes of these strains can be found in **Appendix I**. The expression plasmid, pGS202 carries the *suc*C and *suc*D genes under the control of a $\lambda P_L P_R$ promoter [11]. The recombinant plasmids used in this study is illustrated schematically in Figure 2.1. The nucleotide sequence around the start codon of *suc*D was altered to include an *Nde*I* restriction site. The site-directed mutagenesis protocol of Zoller and Smith [12] was followed with modifications by Kunkel *et al.* [13]. The oligonucleotide sequence is shown in **Appendix II #1**. Mutations were made in M13mp18 derivatives, identified by restriction endonuclease analysis and confirmed by dideoxynucleotide sequencing [14]. Genetic manipulations were performed according to standard protocols [15]. Restriction endonucleases and additional DNA modifying enzymes were purchased from Bethesda Research Laboratories and New England Biolabs. All other chemicals and reagents were bought from Sigma and British Drug House.

B. EXPRESSION AND CELL FRACTIONATION

Cultures of TK3D18 cells containing the pGS plasmids were grown in LB/KCl broth supplemented with ampicillin (50 μg/ml). See **Appendix III** for media composition. Cultures were incubated at

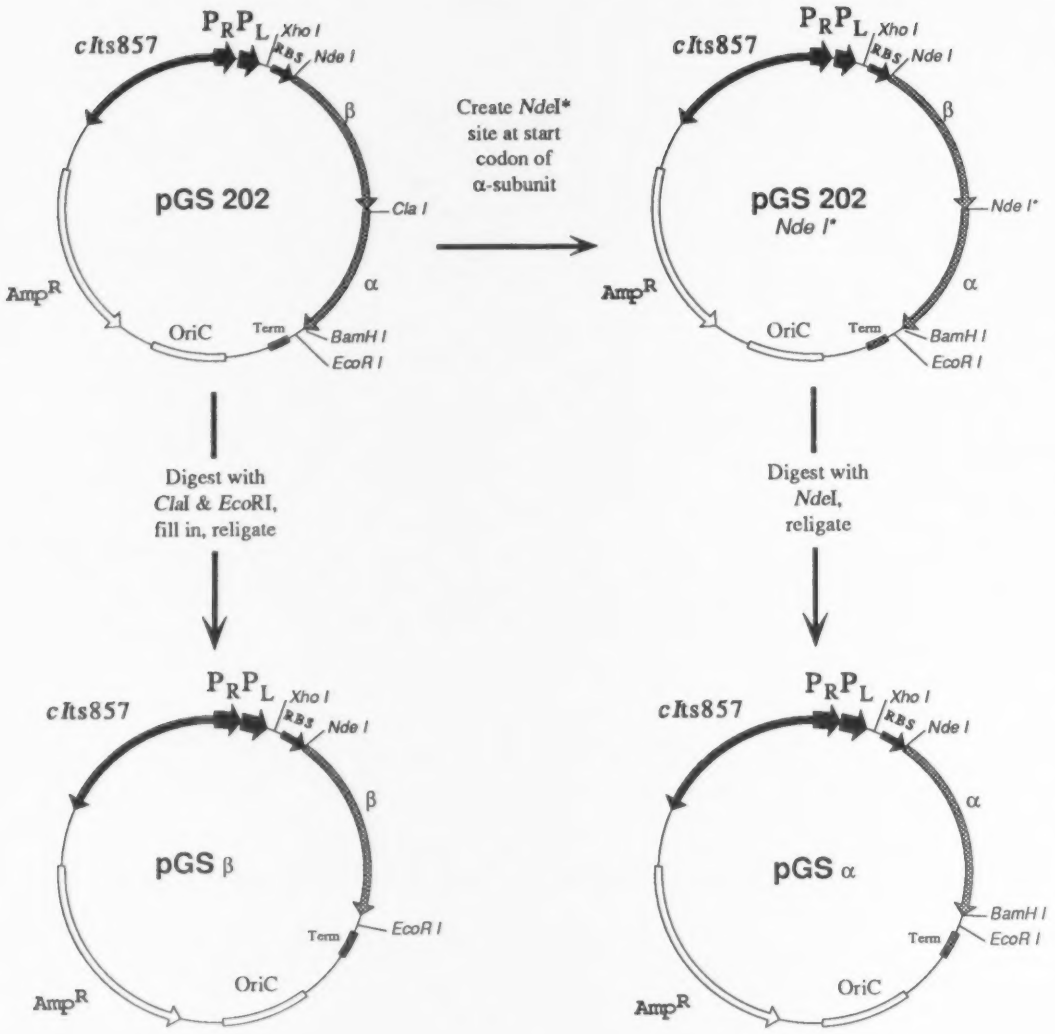

Figure 2.1. Construction of plasmids for the production of subunits of succinyl-CoA synthetase. The expression plasmid, **pGS202** carries the genes for the α- and β-subunits of *E.coli* succinyl-CoA synthetase. The genes are under the control of a $\lambda P_L P_R$ promoter and the translation initiator sequence (**RBS**) of the *atp*E gene. Expression plasmids capable of producing either the α- or the β-subunit of the bacterial enzyme (**pGSα, pGSβ**) were created by deleting one or other of the genes from pGS202. The nucleotide sequence around the start codon of the gene for the α-subunit was altered to include an ***Nde*I*** restriction site.

30° C with aeration. When the A_{600} reached ~0.6, expression was induced by raising the temperature of incubation to 42° C. One ml samples of culture were centrifugation after 5 hours. Cell pellets were resuspended in 0.2 volume (relative to culture volume) of ice cold sonication buffer: 100 mM Tris HCl (pH 7.4), 100 mM potassium phosphate, 100 mM KCl, 1 mM EDTA. Cell suspensions were sonicated and the resulting lysates were centrifuged at 15,000xg for 5 minutes. The supernatants were discarded and the pellets were washed in a further 0.2 volume of sonication buffer. The suspensions were then centrifuged at 100xg and the pellets were solubilized in 0.1 volume of denaturing buffer: 6.0 M GuCl, 50 mM Tris.HCl (pH 7.4), 0.1 mM EDTA, 0.1% β-mercaptoethanol.

C. Refolding of denatured subunits

Solubilized protein pellets from the fractionation described above were left standing at room temperature overnight to effect complete denaturation. The samples were clarified by centrifugation at 15,000xg for 30 min to remove any cell debris. The denatured subunits were refolded using a modification of the method of Pearson and Bridger [2]. Equimolar mixtures of the subunits were diluted into refolding buffer (100 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, 0.5 mM ATP, 5 mM DTT) such that the final concentration of denaturant (GuCl) was reduced to 0.2 M or less. Protein concentrations in the refolding mixtures were in the range of 50-100 μg/ml. Refolding was allowed to proceed for 1 hr. at 20 °C. Samples were assayed for enzymatic activity using the

spectrophotometric method [16]. Chromatography media used in the purification of the enzyme were supplied by Pharmacia and Biorad. The preparation and use of these reagents are described in **Appendix IV**.

III. RESULTS

A. Production of the individual subunits

Expression plasmids capable of producing either the α- or the β-subunit of the bacterial enzyme (pGSα, pGSβ) were created by deleting one or other of the genes from pGS202 (Fig. 2.1). Cultures of TK3D18 cells containing each of these vectors were grown to an A_{600} of ~0.6 and induced to express their respective genes. Samples were taken at varying times following induction and analyzed by SDS-PAGE for the amounts of subunit protein produced (Fig. 2.2). High levels of both the α- and the β-subunit accumulated after induction regardless of whether the subunits were produced alone (pGSα and pGSβ) or together (pGS202). The amounts of subunit protein found at different time points after induction were comparable between the two systems. The subunit protein accounted for as much as 25 % of total cellular protein after 5 hours of induction. Longer periods of expression led to a substantial increase in the host cellular protein levels and resulted in cells that were more resistant to sonication. For these reasons, 5 hours of expression was deemed to be optimal for the production of the two subunits.

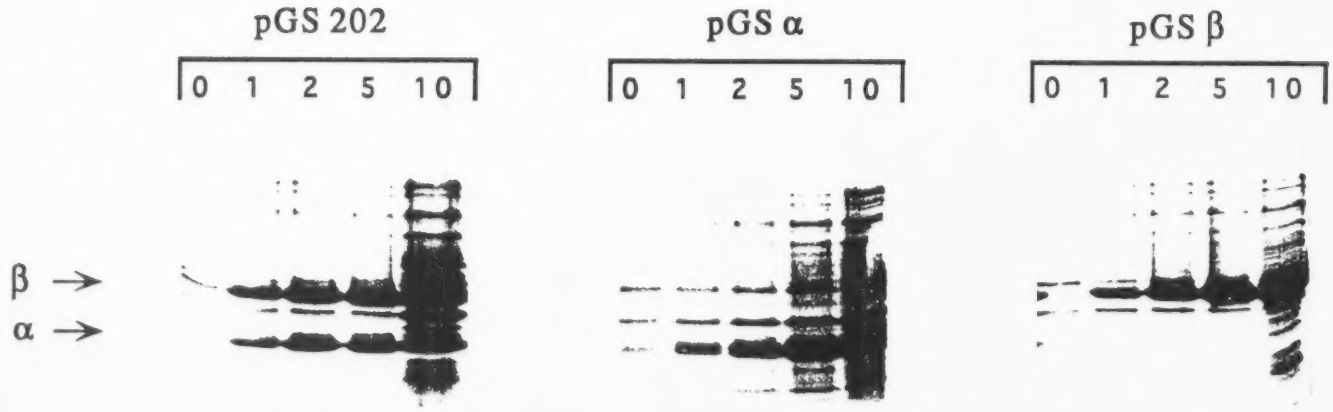

Figure 2.2. Production of subunits of *E. coli* succinyl-CoA synthetase. TK3D18 cells containing the expression plasmids, pGS 202, pGS α and pGS β were grown at 30°C to an A_{600} of 0.5. Expression was induced by raising the temperature to 42°C. See **Materials and Methods** for details. Samples were withdrawn from the cultures at 0, 1, 2, 5 and 10 hours following the induction, centrifuged and the cell pellets were lysed in SDS-PAGE running buffer. Samples were analyzed by SDS-PAGE followed by Coomassie staining.

B. Subunits form insoluble aggregates

Cultures of TK3D18 cells containing each of the pGS expression plasmids were induced for 5 hours. Cells were harvested by centrifugation, resuspended in sonication buffer and sonicated. Lysates were centrifuged at 15,000xg for 5 minutes after which the supernatant and pellet fractions were analyzed by SDS-PAGE (Fig. 2.3). Greater than 75 % of the subunit protein produced from cells containing pGS202 was found in a soluble form and represented the bulk of enzyme activity arising from such expression. The remaining 20-25 % found in the pellet was comprised primarily of the β-subunit. In contrast, the majority of the subunit protein resulting from expression of either pGSα or pGSβ was found in the pellet fractions. Thus, both α- and β-subunits form aggregates when produced in the absence of a companion subunit with which to assemble a soluble enzyme. The insoluble β-subunit produced from pGS202 containing cells likely resulted from unequal expression of the two genes. High levels of expression may compromise the coordination system such that the product of the upstream gene is synthesized in greater amounts. Similar findings were reported by Buck and Guest [11].

C. Purification of the subunits

The characteristics of these subunit aggregates were typical of inclusion bodies [17]. That the subunits accumulated as insoluble aggregates greatly simplified their subsequent purification. Aggregates comprised of either α- or β-subunits were resuspended in buffer and subjected to a range of different centrifugation conditions

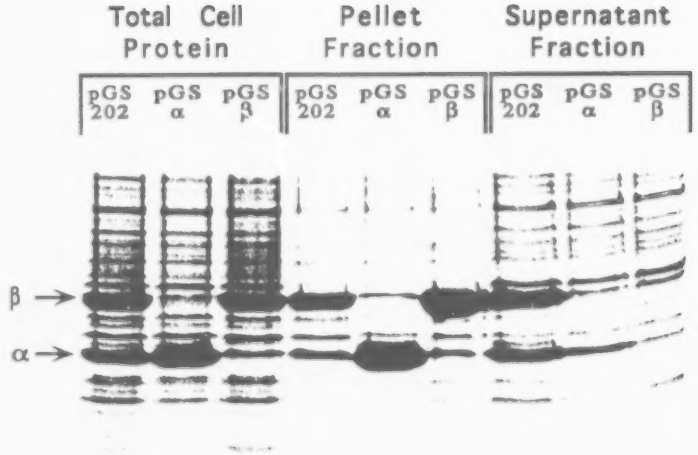

Figure 2.3. Fractionation of lysates of *E. coli* containing subunits of succinyl-CoA synthetase. Cultures of TK3D18 carrying the expression plasmids, pGS202, pGSα and pGSβ were induced to express subunits of the enzyme for 5 hours. Cell pellets and supernatant and pellet fractions derived from the centrifugation of sonicated lysates were resolved by SDS-PAGE.

(Fig. 2.4*a*). Over 90 % of the aggregates of either subunit were found in the pellet following a 10 minute centrifugation at 100xg. Higher centrifugation speeds resulted in greater amounts of contaminating host protein in the pellet. Thus, inclusion of a 100xg centrifugation step following the initial fractionation had the effect of removing cellular contaminants from the subunits. Aggregates obtained from the 100xg fractionations were tested for their solubility in solutions containing different concentrations of GuCl (Fig. 2.4*b*). Both subunits were readily solubilized in a 1.8 M GuCl solution, whereas the aggregates were only partly solubilized in 1.2 M GuCl. Following an overnight incubation at room temperature in 1.8 M GuCl any remaining insoluble material could be removed by centrifugation. The concentration of the GuCl was adjusted to 6.0 M to effect complete unfolding of the protein. In this way we were able to obtain preparations of both subunits that were judged to be over 95 % pure.

D RECONSTITUTION OF ENZYME ACTIVITY

Subunits purified from the inclusion bodies were capable of reconstituting enzyme activity. A series of small scale (100 µl) refolding reactions was carried out as outlined in **Materials and Methods**. Reaction mixtures were set up in which increasing amounts of α-subunit were added to a fixed quantity of β-subunit. Optimal reconstitution of enzyme activity was achieved with equimolar amounts of the two subunits (Fig. 2.5). The specific activity of reconstituted mixtures reached a peak at a 1:1 molar ratio of added subunits but decreased as excess α-subunit was added. No

Figure 2.4. Purification of subunits from inclusion bodies. ***(a)*** Lysates of cells induced to produce α- or β-subunits of the enzyme for 5 hours were subjected to differential centrifugation to determine their sedimentation characteristics. Each lysate was divided into five equal samples. Each sample was subjected to one of the following low speed **(L)** spins for 5 min.: 50, 100, 500, 2500 or 5000 xg. The supernatant from the low speed spin was then centrifuged at 12500xg **(H)**. The pellets from each centrifugation were resolved on SDS-PAGE. ***(a)*** Inclusion bodies centrifuged at 500xg were resuspended in sonication buffer and divided into five equal samples. Each sample was treated for 1 hour by the addition of GuCl at one of the following concentrations: 0.0, 0.6, 1.2, 1.8 or 2.4 M. The samples were centrifuged for 30 min at 12500xg and the pellets of insoluble material were analyzed by SDS-PAGE.

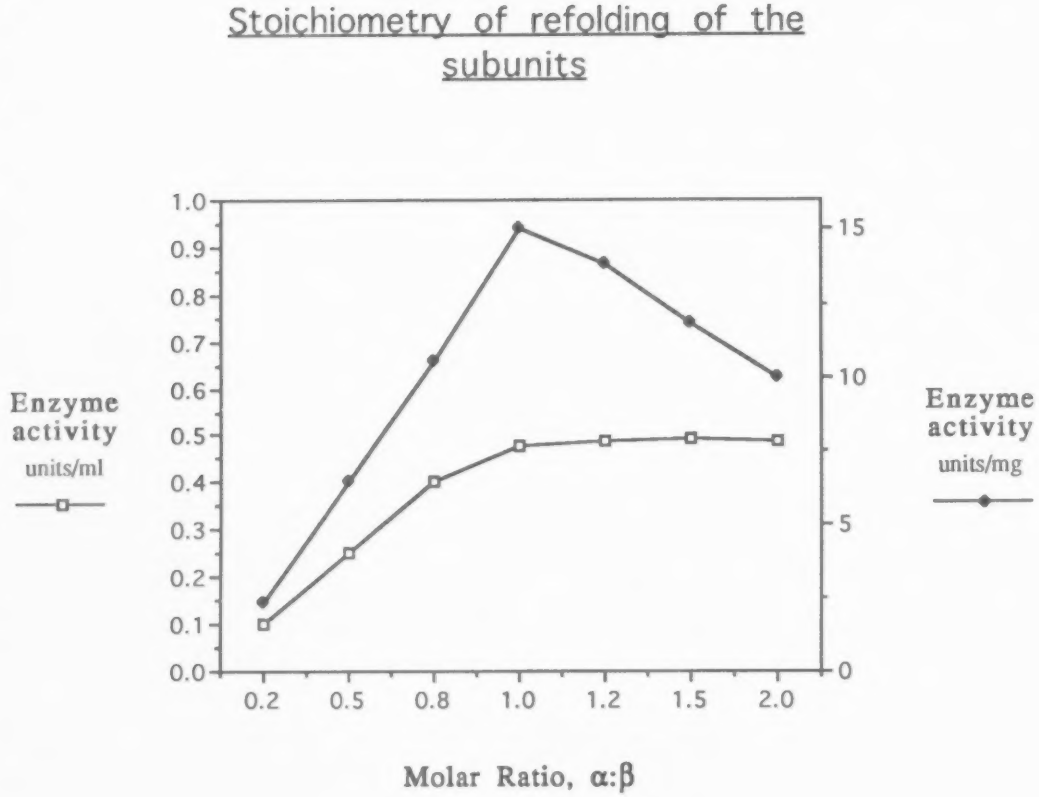

Figure 2.5. Stoichiometry of assembly from denatured subunits. Refolding mixtures were set up to include a constant molar amount of the β-subunit (65 µg/ml) and increasing amounts of the α-subunit. The subunits were diluted into the refolding pots and allowed to renature and assemble over one hour. After completion of the refold, samples were assayed for enzymatic activity.

activity was detected from renaturation of the individual subunits. The highest specific activity achieved from these reconstitutions was 15 units/mg. Given that the specific activity observed with the enzyme purified by conventional means is in the range of 40 units/mg, this represents a reconstitution efficiency of 40 %. The efficiencies obtained in this study are similar to those reported in earlier work [2,3]. A conclusion from this is that the subunits prepared from bacterial lysates are not significantly damaged and behave as well as those prepared from a highly purified enzyme. Most probably, subunit proteins are afforded a degree of protection against cellular damage by forming compact inclusion bodies.

E. Purification of the reconstituted enzyme

In calculating efficiency of reconstitution, it is assumed that the reaction mixtures are composed of both fully active enzyme molecules and misfolded inactive components. This assumption can be verified by purifying the active species from a reconstituted mixture. A large scale (100 ml) reconstitution was performed from which the active enzyme was purified using a series of chromatographic steps. Details of the purification procedures are given in Figure 2.6. The reconstitution mixture was first centrifuged to remove aggregated material. The clarified supernatant was concentrated to one twentieth of its volume. The sample was passed through a Sephacryl S300 gel filtration column. The elution profile revealed the active species to be of a size consistent with that of a tetrameric ($\alpha_2\beta_2$) enzyme. The active component was further purified on hydroxyapatite and by affinity chromatography with affigel blue.

(a)

Purification of reconstituted enzyme from refold reactions

Purification step	Total recovered activity (units)	Specific activity (units/mg)
Refold	110	13.8
S300	95	15.2
Hydroxyapatite	78	16.5
Affigel Blue	59	37.0

(b)

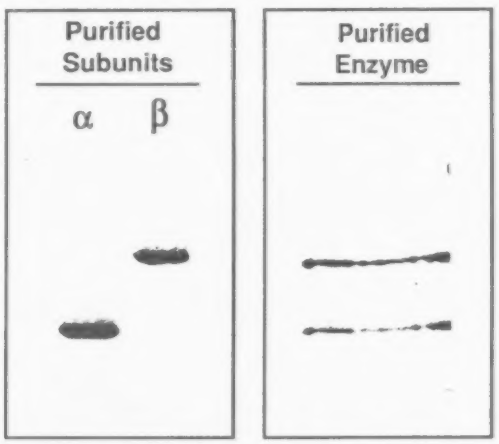

Figure 2.6. Purification of the active reconstituted enzyme. ***(a)*** A 100 ml reconstitution was carried out with the *E. coli* α- and β-subunits purified from inclusion bodies. The active component was purified through a series of chromatographic steps. A concentrated sample of the refold was gel filtrated through Sephacryl S300. The pool of active fractions was subsequently purified by chromatography with hydroxyapatite using a 10-400 mM potassium phosphate gradient and by affinity chromatography through Affigel Blue using a 0-2 M KCl gradient. The specific activity of the enzyme preparation at each stage is shown here. ***(b)*** The subunits used in the reconstitution and the purified reconstituted enzyme were resolved on SDS-PAGE to assess their purity.

Activities were assayed at each step, the results of which are presented in Figure 2.6*a*. Specific activities following the first two purification steps remained relatively low. It was only after the enzyme mixture was applied to the affigel blue matrix that the active species was effectively purified from inactive components. The specific activity of the final enzyme preparation was 37 units/mg, a value comparable to that observed for the native enzyme. This finding supports the original assumption with regard to the composition of the reconstituted mixtures. Thus, 40 % of the subunits in the reconstitution mixtures assemble into a fully active enzyme. Figure 2.6*b* shows the electrophoretic analysis of the purified enzyme alongside the subunits used in the reconstitution.

IV. DISCUSSION

The original methods employed to study the refolding and assembly of SCS from its individual subunits involved preparative procedures that were both time consuming and labor intensive and required large quantities of materials. Specifically, the enzyme was purified from source, denatured in urea and the individual subunits were separated by gel filtration [2,3]. Purification of the enzyme in the amounts required for such experiments involved several chromatographic steps with continual monitoring and typically take over two weeks. The preparation of individual subunits from the denatured enzyme involved separation by gel filtration and was problematic because of their similar molecular masses. Consequently,

this step resulted in unnecessary losses and often needed to be performed twice.

The development of a recombinant DNA-based method for producing subunits of the enzyme represents a significant improvement on the former method. Predetermined quantities of individual subunits can be prepared quickly, without losses and with minimal materials using this procedure. Subunits produced by recombinant means can be assembled into active enzyme with efficiencies similar to those achieved with the subunits separated from purified enzyme. This method, in combination with a mutagenesis approach, will facilitate a more detailed examination of the folding and assembly pathways.

In contrast to the bacterial system, assembly of the mammalian enzyme is less well characterized. Reconstitution of the mammalian enzyme has been demonstrated following renaturation of a mixture of the isolated subunits [18]. The process of assembly is dependent on the presence of either glycerol or polyethylene glycol. This may be reflective of the unusually high protein concentrations found in the mitochondrial matrix. Further studies on the biogenesis and assembly of the mammalian enzyme would greatly benefit from the use of these recombinant-DNA methods.

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Chapter 3

Conditions for the production of precursor and mature forms of the mammalian α-subunit

I. INTRODUCTION

The most active preparations of the enzyme, succinyl-CoA synthetase, have been obtained from *E. coli* [1-3] and pig heart [4-6]. Consequently these are the best characterized forms of the enzyme. Although similar in catalytic properties, significant differences are found between these two forms of the enzyme (Chapter 1 Table 1.I). The *E. coli* enzyme is a nondissociating $\alpha_2\beta_2$ tetramer, whereas the pig heart enzyme is a nonassociating $\alpha\beta$ dimer [7,8]. Both enzymes can be assembled, *in vitro*, from mixtures of previously separated subunits [9-12]. While the *E. coli* enzyme reconstitutes in simple phosphate buffers [9,10], assembly of the pig heart enzyme depends on the presence of 15 % glycerol or polyethylene glycol [12]. This may be reflective of the different environments in which the two enzymes fold. The bacterial enzyme is assembled in the cytosol of the cell, whereas the eukaryotic enzyme is found in the mitochondrial matrix.

The biogenesis of mammalian succinyl-CoA synthetase includes a step not found on the pathway to assembly of the bacterial enzyme. Subunits of the mammalian enzyme must be imported into mitochondria prior to assembly. The subunits are encoded by nuclear genes and are synthesized in the cytosol as higher molecular weight species called precursors [13,14]. These precursors have N-terminally located signal sequences that serve to direct the translocation of the subunits across the mitochondrial membranes. The signal sequences are removed during the translocation process, releasing the mature subunits in the matrix where they assemble into the dimeric enzyme

[15]. Gaining insight into how this translocation is achieved is of central importance to understanding biological compartmentalization of cellular functions in eukaryotes.

Precursors do not accumulate in eukaryotic cells under normal physiological conditions. However, a cDNA (λSCS19-1) encoding the precursor form of the α-subunit of rat liver succinyl-CoA synthetase has been isolated [13]. Expression of this sequence in bacterial cells would allow for its purification and permit investigations on the translocation of this subunit of the mammalian enzyme. Conditions for the production of the precursor and its mature counterpart in bacterial cells are described in the following pages.

II. MATERIALS AND METHODS

A. Bacterial strains vectors and reagents

The following strains of *E. coli* were used in these studies: JM109 and GM48 were employed for molecular cloning work; expression was carried out in DHI. GM48 was used to prepare DNA that was unmethylated at *dam* and *dcm* sites. The genotypes of these strains can be found in **Appendix I**. The plasmids, pGP1-2, pT7-6 and pT7-7, were a gift from Dr. Stan Tabor of Harvard. pTZ19 was purchased from Pharmacia. Restriction endonucleases and additional DNA modifying enzymes were purchased from Bethesda Research Laboratories and New England Biolabs. Chemicals and reagents were bought from Sigma or British Drug House. [^{3}H]uridine was supplied by Amersham Canada.

B. VECTOR CONSTRUCTION AND MUTAGENESIS

The plasmid, pUC19 [16], was modified by insertion of a translation initiation sequence generating pUC19W$_9$ (Fig. 3.1*a*). Expression constructs were created in this plasmid, then, transferred as *Hin*DIII/*Eco*RI fragments to the expression vector pT7-6 (Fig. 3.1*b*) or to pTZ19. pT7-7 contains its own translation initiation sequence. Thus, recombinants of this vector were generated by direct sequence transfer.

The open reading frame of λSCS19-1 (Fig. 3.1*c*) was cloned into pUC19W$_9$ as an *Nco*I/*Eco*RI fragment. The *Nco*I site was blunt-end ligated to the *Bam*HI site in the vector. Partial treatment of the *Nco*I and *Bam*HI sites with mung bean nuclease generated a plasmid in which the open reading frame of λSCS19-1 was shifted by one nucleotide. This new reading frame contained two stop codons, located 115-120 bp downstream from the ATG initiator. These two stop codons were removed by site-directed mutagenesis using oligonucleotide #2. Sequence of oligonucleotides can be found in **Appendix II**. In addition, the reading frame was corrected 165 bp downstream from the ATG by digesting with *Kpn*I, filling in the ends and religating. The λSCS19-1 sequence was fused in frame at position 891 bp (see Fig. 3.1*c*) to the 5' end of the *lac*Z gene in pUC19W$_9$.

A *Kpn*I/*Eco*RI fragment of λSCS19-1 was ligated to the same sites of pUC19W$_9$. Mutagenesis with oligonucleotides #3 and #4 at the 115 bp position of λSCS19-1 created a second *Nco*I* site and a unique *Cla*I* site, respectively. Sequence of oligonucleotides can be found in **Appendix II**. The *Nco*I* site was blunt-end ligated to the *Bam*HI site of pUC19W$_9$ as above. λSCS19-1 containing the unique

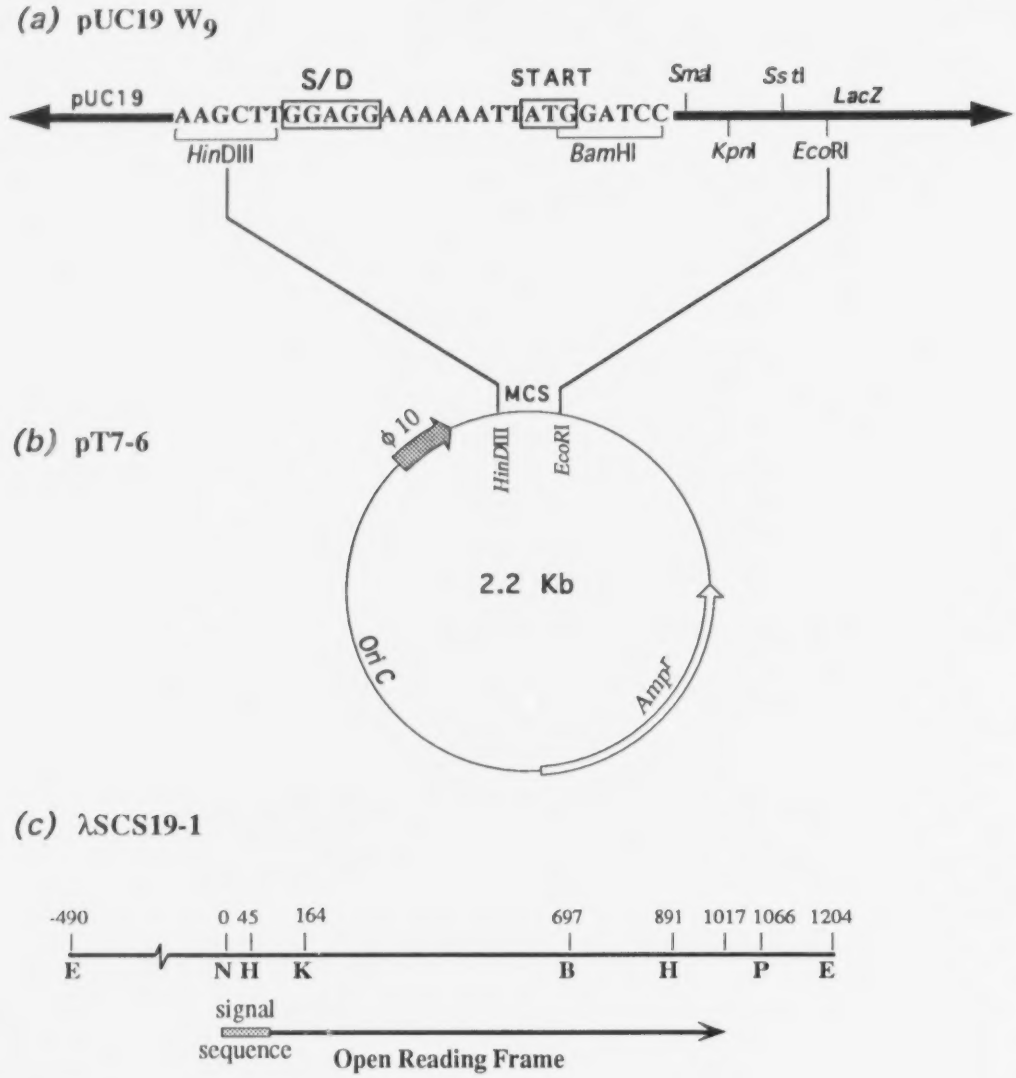


Figure 3.1. Plasmids and restriction site map of λSCS19-1. ***(a)*** pUC19W$_9$ showing the translation initiation sequence cloned between the *Hin*DIII and *Bam*HI sites. Insertion of genes between the *Bam*HI and *Eco*RI sites renders them capable of translation in *E. coli* . ***(b)*** Cassettes were transferred as *Hin*D III/*Eco*R I fragments into the corresponding sites in pT7-6, thus placing them under the control of the T7*Φ10* promoter. ***(c)*** The restriction map of λ SCS19-1, a rat liver cDNA clone. The reading frame, which includes the signal sequence, runs from the *Nco*I Site at position 0 to position 1017. Restriction sites are as follows: N, (*Nco*I, 0); E, (*Eco*RI, -490 & 1204); H (*Hin*PI, 45 & 891); K, (*Kpn*I, 164); B, (*Bam*HI, 697); P, (*Pst*I, 1066).

*Cla*I* site was used to fuse the signal sequence of the rat liver cDNA, in frame, to the sequence encoding the *E. coli* α-subunit. Plasmids capable of producing the *E. coli* subunits, either alone or in combination, have been described in Chapter 2. These constructs were transferred directly to pT7-6.

All genetic manipulations were performed as described in Maniatis *et al.* [17]. Site-directed mutagenesis was performed by the method of Zoller and Smith [18] with modifications by Kunkel *et al.* [19]. Mutations were made in M13mp18 derivatives, identified by restriction digest analysis and confirmed by dideoxynucleotide sequencing [20].

C. T7-INDUCED EXPRESSION

The plasmid, pGP1-2, contains the T7 RNA polymerase gene under a heat-inducible promoter. Sequences to be expressed were placed under the control of a T7 *ϕ10* promoter in pT7-6, pT7-7 or pTZ19. DHI cells containing a combination of pGP1-2 and a pT7 recombinant were grown up at 28 °C in LB medium supplemented with ampicillin (50 μg/ml) and kanamycin (40 μg/ml). See **Appendix III** for media composition. Induction is achieved by raising the temperature to 42 °C

The T7 polymerase/promoter system allows one to specifically label the plasmid encoded protein, thus, increasing the sensitivity of detection [21]. Since host RNA polymerase can be inactivated by rifampicin, transcripts produced under the control of the T7 promoter can be selectively enriched allowing exclusive labeling of translated proteins with [^{35}S]methionine. Thus, even poorly

expressed genes can be examined by subjecting total cell lysates to SDS-PAGE followed by autoradiography. Procedures for exclusive labeling of plasmid-encoded proteins using this T7 RNA polymerase/promoter system were essentially those described by Tabor & Richardson [21]. β-Galactosidase activity resulting from expression of *lac*Z fusions was measured according to the procedure of Miller [22].

D. RNA ISOLATION

To measure levels of transcript produced in the expression systems, cells were grown at 28^0 C with aeration in LB medium containing ampicillin and kanamycin. When the culture reached an A_{600}=0.5, one ml of culture was induced by shifting to 42^0 C for 15 minutes. Rifampicin was added tc a final concentration of 0.4 mg/ml and the culture was incubated for a further 30-45 minutes to deplete host RNA. Cells were pulsed with [^{3}H]uridine (10 μCi/ml) for 10 minutes. The RNA was then isolated by the hot phenol/SDS procedure of von Gabain *et al.* [23]. Tritium incorporation was measured with a liquid scintillation counter.

III. RESULTS

A. THE PRECURSOR FAILS TO ACCUMULATE

The open reading frame of λSCS19-1 (*Nco*I/*Eco*RI) was placed downstream from a strong translation initiation sequence in pT7–6 (see **Materials and Methods**). Cultures of DHI cells containing this recombinant and pGP1-2 were grown up at 28 °C. Cultures were

induced at 42^0 C when the A_{600} reached 0.5 and were pulse-labeled with [^{35}S]methionine. The cell lysate from a 10 minute labeling period was resolved by SDS-PAGE followed by autoradiography. No labeled protein was detected in the lysate (Fig. 3.2, lane 3), whereas the expression of the *E. coli* genes resulted in two well labeled subunits (Fig. 3.2, lane 1).

Since the mature portion of the rat liver α-subunit is homologous to the *E. coli* α-subunit [13], the signal sequence was suspected in causing the failure to accumulate. Other workers have reported similar difficulties in producing precursor proteins that were overcome by deletion of all or part of the signal sequence [24-27]. A pT7-6 recombinant containing the *Kpn*I/*Eco*RI fragment of λSCS19-1 produced a labeled rat liver α-subunit (Fig. 3.2, lane 4). Thus, deletion of the first 165 bp of the λSCS19-1 open reading frame allowed the efficient expression of the mature α-subunit of the rat liver enzyme. The size of the subunit produced was consistent with that expected from the sequence and was immunoprecipitable with polyclonal antibodies directed against the purified enzyme (Fig. 3.2, lane 8).

B. The sequence encoding the signal peptide blocks expression

It is conceivable that the presence of a signal peptide in the precursor may cause the protein to become protease-sensitive [28]. Thus, failure to accumulate protein could be rationalized by a rapid degradation of the precursor. A frame shift in the sequence encoding the signal peptide would, therefore, be expected to allow for efficient expression. The reading frame of the first 165 bp of the λSCS19-1

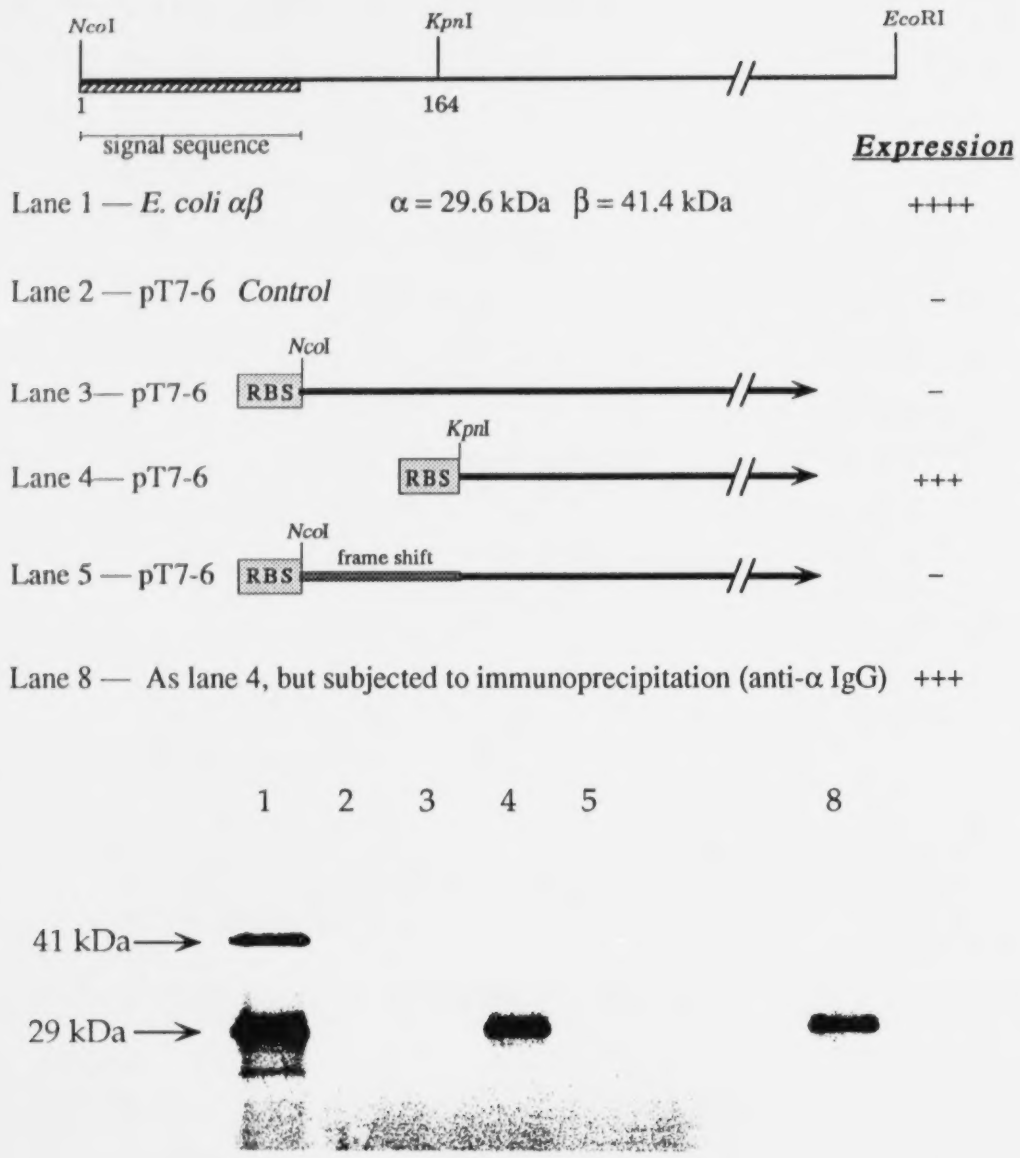


Figure 3.2. Expression of the open reading frame of λSCS19-1 in pT7-6. Autoradiograph of total cell lysates subjected to SDS-PAGE. The clones schematically illusrated above were examined for their ability to produce protein using the exclusive labelling technique. Lane 1 shows *E. coli* succinyl-CoA synthetase α- and β-subunits (29 kDa and 41 kDa, respectively). Lane 2 represents a control pT7-6 without insert. Lanes 3, 4 and 5 are different constructs made in pT7-6 with sequences from the rat liver cDNA, λSCS19-1.

was shifted by one nucleotide (see **Materials and Methods**). DHI cells containing this recombinant were pulse-labeled as before and resolved on SDS-PAGE. The frame shift failed to allow expression of the λSCS19-1 sequence (Fig. 3.2, lane 5). The result from this frame shifting experiment argues against proteolytic degradation playing a role. Although not excluded, the possibility that the two different N-terminal protein sequences would produce such similar effects was seen as unlikely. Thus, the result is more in favor with the nucleotide sequence being the cause of failure in expression.

Deletions of 45 and 115 nucleotides from the 5' end of the λSCS19-1 open reading frame were made (Fig. 3.3). pT7-6 recombinants carrying these deletions were assessed for expression. Removal of the first 45 nucleotides of the sequence failed to allow expression (Fig. 3.3, lane 3), whereas deletion of 115 nucleotides resulted in the appearance of a labeled α-subunit (Fig. 3.3, lane 4). This suggested that the sequence residing between residues 45 and 115 was responsible for blocking expression. However, λSCS19-1 sequences carrying an internal deletion of these 70 nucleotides also failed to express (Fig. 3.3, lane 5). The results can be interpreted as indicating that sequences both within the first 45 nucleotides and within the following 70 nucleotides can prevent expression, independently.

The first 115 bp of the λSCS19-1 open reading frame were fused, in frame, to the 5' end of the *E. coli* α-subunit gene. While the *E. coli* α-subunit is easily detected in labeled cell lysates, the fusion displayed the usual absence of protein (Fig. 3.3, lanes 7 and 8).

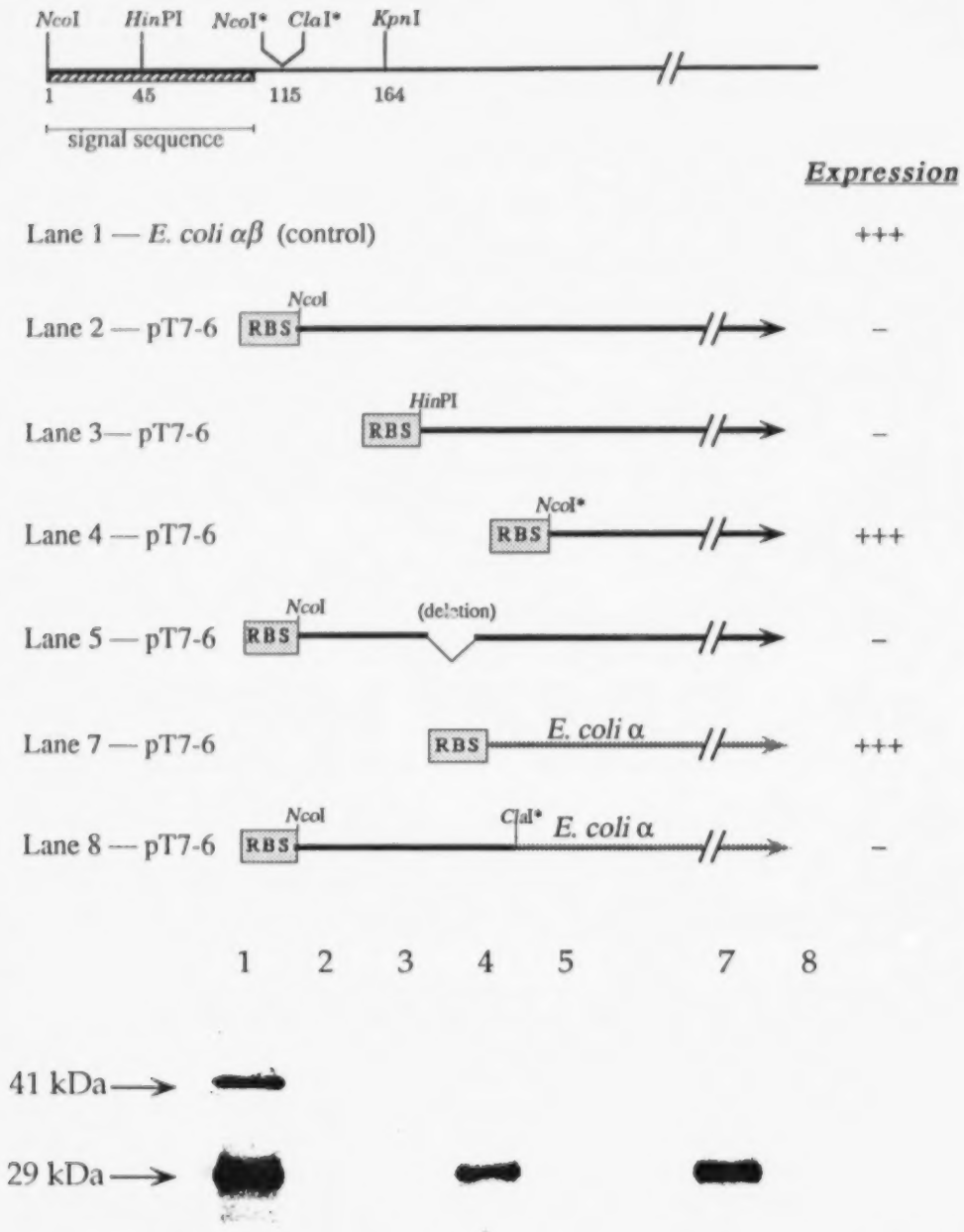
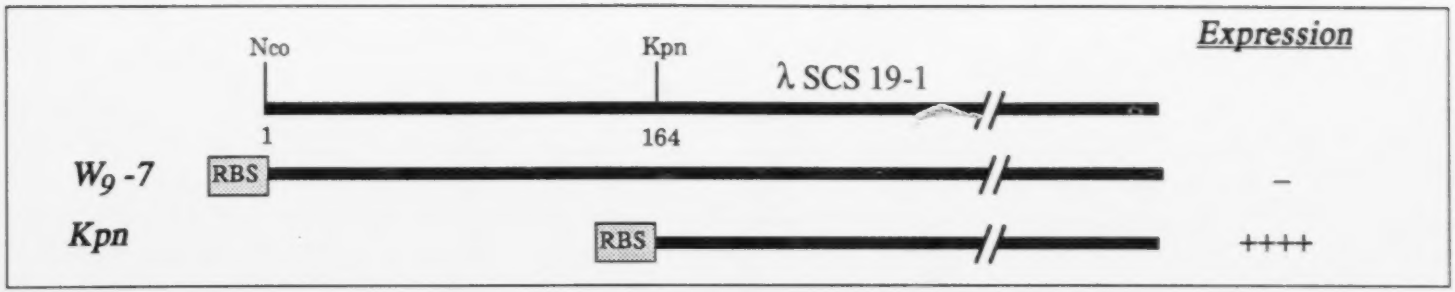


Figure 3.3. Examination of sequences encoding the signal peptide of the α-subunit. Autoradiograph of lysates subjected to SDS-PAGE. The clones schematically illustrated above were examined for their ability to produce protein. Deletion were made in the 5' end of the λSCS19-1 open reading frame. Total cell lysates were resolved on SDS-PAGE followed by autoradiography.

Thus, the presence of these sequences at the 5' ends of other genes blocks their expression also.

C. Inhibition is at the level of translation

Absence of expression has been attributed to the nucleotide sequence at the 5' end of the λSCS19-1 open reading frame. Failure to show expression could result from a block in either transcription or translation. Most expression systems in common use are based on the ability of a strong promoter to drive transcription with little regard to the nature of the downstream gene. Nevertheless, transcription rates were assessed. Transcription efficiency was measured by the ability of T7 expressing cells to incorporate [^{3}H]uridine into RNA following rifampicin treatment. Rates of transcription were compared for pT7-6 recombinants containing the complete open reading frame of λSCS19-1 and one in which the first 165 bp were deleted. Both of these incorporated similar levels of label into RNA following induction (Fig. 3.4). The levels were comparable to those of the positive control (pT7-6). This incorporation was resistant to rifampicin treatment, showing it to be T7-induced, whereas the negative control, pGP1-2 alone, showed very low levels of incorporation. These results confirm that the transcription rates are unaffected by the presence of sequence at the 5' end of λSCS19-1. Since transcription of the foreign genes is not affected, failure to show expression must, therefore, result from a block in translation.



Plasmid Background	DPM of ^{3}H-uridine in RNA Preparations Following Induction	
	– Rifampicin	+ Rifampicin
pGP1-2	59800	1000
pGP1-2/pT7-6	60000	72000
pGP1-2/pT7-6 *W97*	56000	69700
pGP1-2/pT7-6 *Kpn*	53000	65000

Figure 3.4. Transcription rates of T7 controlled genes. Comparison of the rates of incorporation of [^{3}H]uridine into newly synthesized RNA in cells containing expression plasmids. Above the table is a scheme showing the two rat liver constructs tested for RNA synthesis. Rates were measured for cells with and without rifampicin treatment. pGP1-2 alone represents the negative control while the positive control contained pT7-6 in addition. Cells were pulsed with [^{3}H] uridine for 10 minutes followed by isolation of RNA. Samples were measured on a scintillation counter.

D THE TRANSLATIONAL APPARATUS IS FUNCTIONAL

If the absence of expression is caused by a block in translation of the message, it would seem appropriate to check that the cellular translational components are not adversely affected in cells containing the message. A series of recombinant plasmids were constructed in pTZ19 (Fig. 3.5). These plasmids are T7 expression vectors that contain a copy of the β-lactamase gene reading in the same direction as the λSCS19-1 sequence. Thus, induction from the T7 promoter controls the expression of both genes. The β-lactamase gene provides an internal control of the translational capability within induced cells. Lower levels of β-lactamase protein were produced from pTZ19 vectors containing λSCS19-1 sequences compared with the parental pTZ19 vector (Fig. 3.5, lanes 1-4). However, there was little difference seen in the β-lactamase levels produced from the various pTZ19 recombinants. The cell's ability to translate other messages is not compromised specifically by the presence of the 5' sequence of λSCS19-1.

E INITIATION OF TRANSLATION IS COMPROMISED

The open reading frame of λSCS19-1 was fused at the 3' end to the α-peptide of *lac*Z in pUC19W$_9$ (Fig. 3.6). Expression of the *lac*Z fusion was monitored in JM109 which carries a deletion of the α-peptide. High levels of β-galactosidase were detected with a recombinant containing the complete 5' end of the λSCS19-1 sequence (Fig. 3.6). These levels were comparable to the activities measured in the positive control, pUC19W$_9$. This was unexpected since a similar construct generated in pT7-6 failed to produce

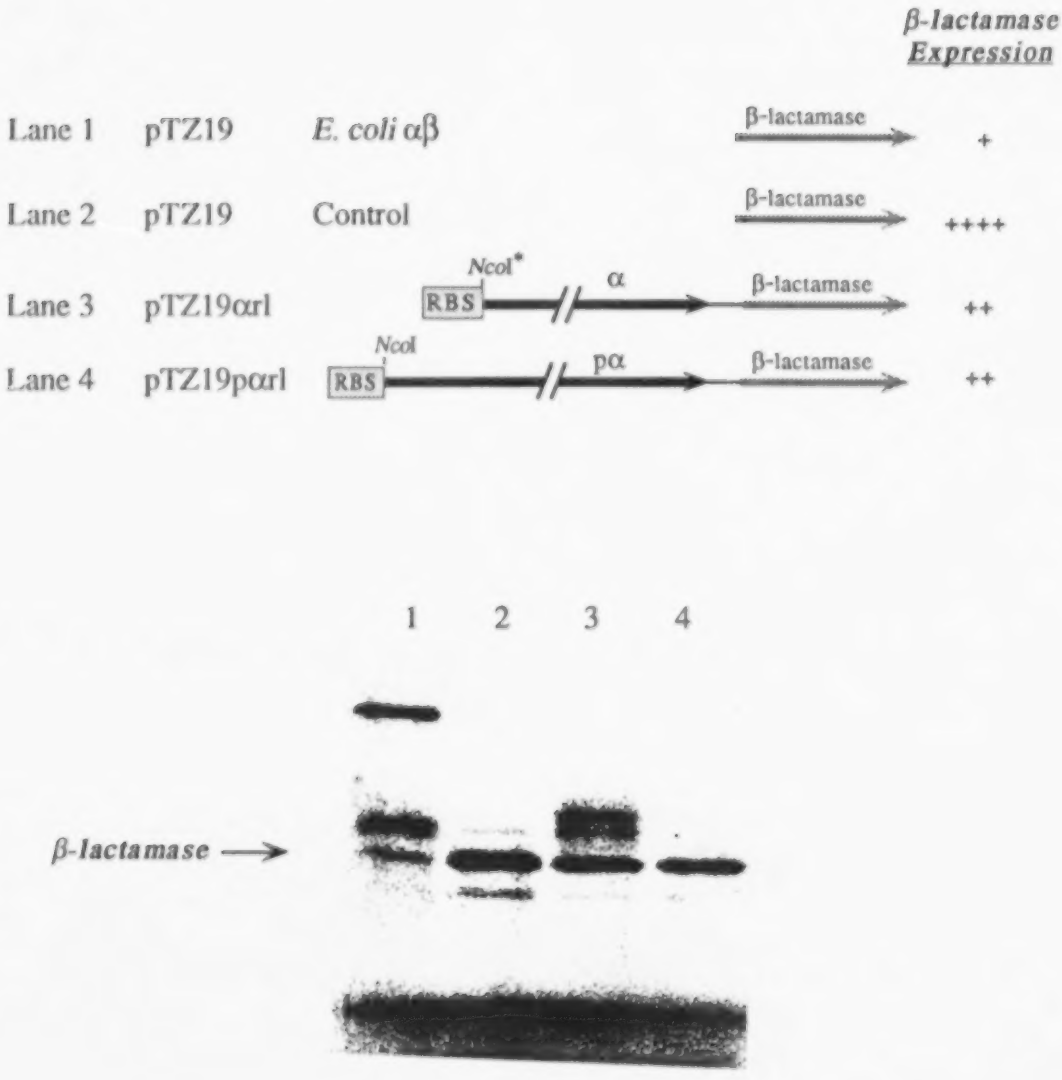


Figure 3.5. Translation of β-lactamase in cells containing pTZ19 plasmids. Autoradiograph of cell lysates resolved by SDS-PAGE. Expression constructs generated in pTZ19 were examined for their ability to produce β-lactamase. A schematic outline of the clones is given above. pTZ19αrl contains the rat liver sequence minus the signal sequence, whereas pTZ19pαrl contains the complete open reading frame. Both the β-lactamase gene and the rat liver sequence are under the control of an upstream T7 promoter.

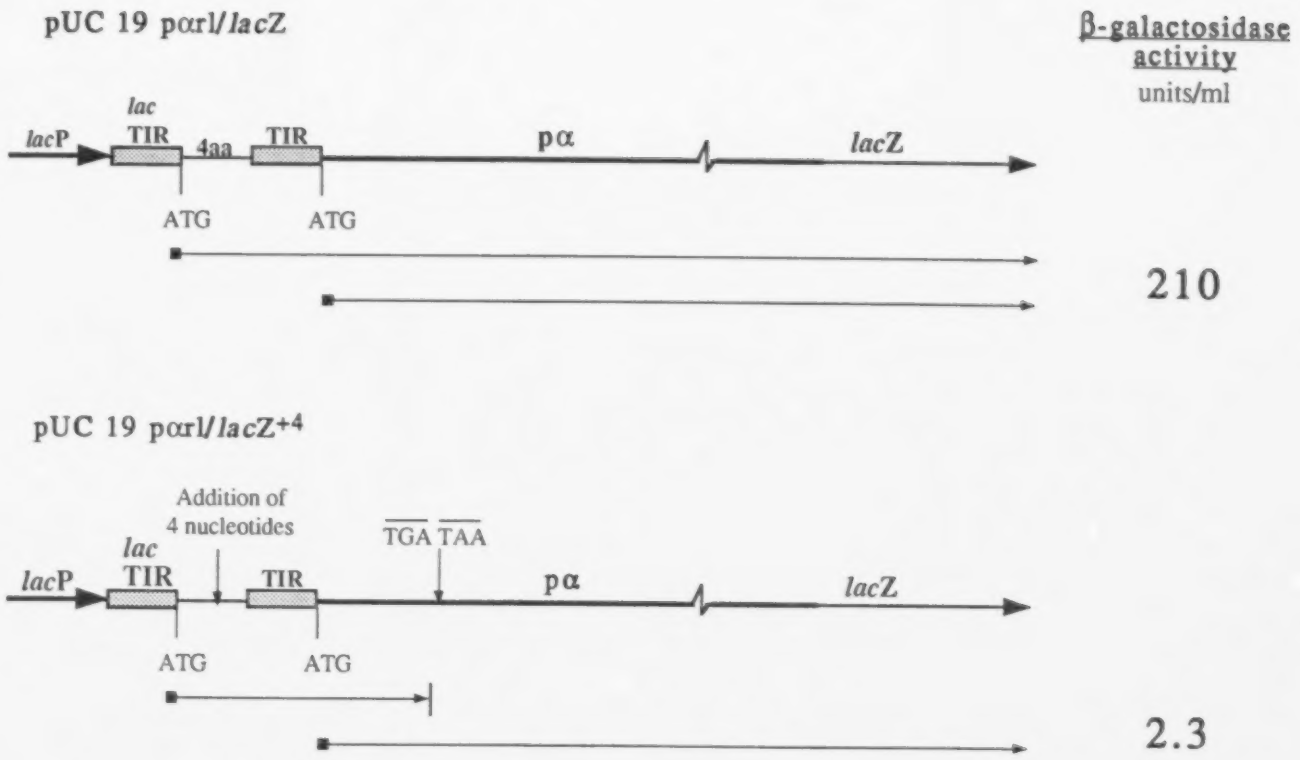


Figure 3.6. The ability of the *lacZ* TIR to mediate expression. *lacZ* fusions were made in the plasmid, pUC19. A portion of the sequence of λSCS19-1 was cloned into the multicloning site of pUC19 such that its reading frame was fused in-frame with the downstream *lacZ* gene. This clone was then modified by cutting and filling in the *HinD*III restriction site causing the addition of four extra nucleotides. Indicated are the two stop codons in the new reading frame.

β-galactosidase activity. A closer examination of the pUC19W$_9$ recombinant showed that the ATG start codon of *lac*Z (not present in pT7-6) was in frame with the downstream initiator of the λSCS19-1 sequence. Thus, translation initiation at the *lac*Z TIR could have produced a functional fusion protein (Fig. 3.6).

A *Hin*DIII restriction site downstream of the *lac*Z initiator but upstream of the λSCS19-1 ATG was digested, filled in and religated. This resulted in insertion of four nucleotides and shifted the reading frame of the *lac*Z TIR-controlled transcripts by one nucleotide. Translation from the *lac*Z TIR would terminate prematurely within the λSCS19-1 sequence and, thus, would not produce a *lac*Z fusion protein. The β-galactosidase levels produced from this frame shift were one hundred-fold lower than the unmodified fusion (Fig. 3.6). Thus, it would appear as though the majority of the β-galactosidase activity detected from the original fusion results from translation initiation at the *lac*Z TIR. This is reminiscent of a report by Kaderbhai *et al.* [27] who found that the addition of a second TIR followed by four amino acid codons upstream from the first overcame a block in translation. The results, therefore, suggest that initiation of translation at the TIR adjacent to the λSCS19-1 sequence is compromised.

F. Secondary structure at the 5' end of the transcript

It would appear from the above results that it might be the proximity of the λSCS19-1 sequences to the TIR that effects translation initiation. Expression of foreign genes is often adversely affected by sequences at the 5' end of the gene. Such effects are

believed to be due to the ability of these sequences to base-pair with the adjacent TIR [29-31]. Variations in translation efficiencies have been seen as a result of such interactions [32-34]. In fact, de Smit & Van Duin [35] point out that very small differences in base-pairing stability can result in dramatic variations in translation efficiency.

Several secondary structures were predicted for the λSCS19-1 sequence. The most interesting of these predictions showed a very stable potential stem-loop at the extreme 5' end of the coding sequence, with a predicted free energy of -22.7 kcal. The AUG start codon was sequestered into this structure (Fig. 3.7*a*). It was thought possible that the involvement of the AUG start codon in such a structure could lead to a block in translation. To test this, six residues centrally located along the 5' arm of the stem were replaced with non-complementary nucleotides while maintaining the encoded amino acid sequence These changes effectively disrupted the secondary structure (Fig. 3.7*b*) but failed to overcome the block in expression.

G. Increasing the distance from the TIR/AUG allows expression

If it is the proximity of the λSCS19-1 sequences that renders the adjacent TIR ineffective, then increasing the distance between the two would be expected to allow for expression. The complete open reading frame of λSCS19-1 was placed 39 nucleotides downstream from the initiation codon in pT7-7 (Fig. 3.8). Thirty-nine nucleotides was thought to provide sufficient spacing on the basis of the *lac*Z fusion results. As a control, the same reading frame was placed immediately adjacent to the ATG in the same vector.

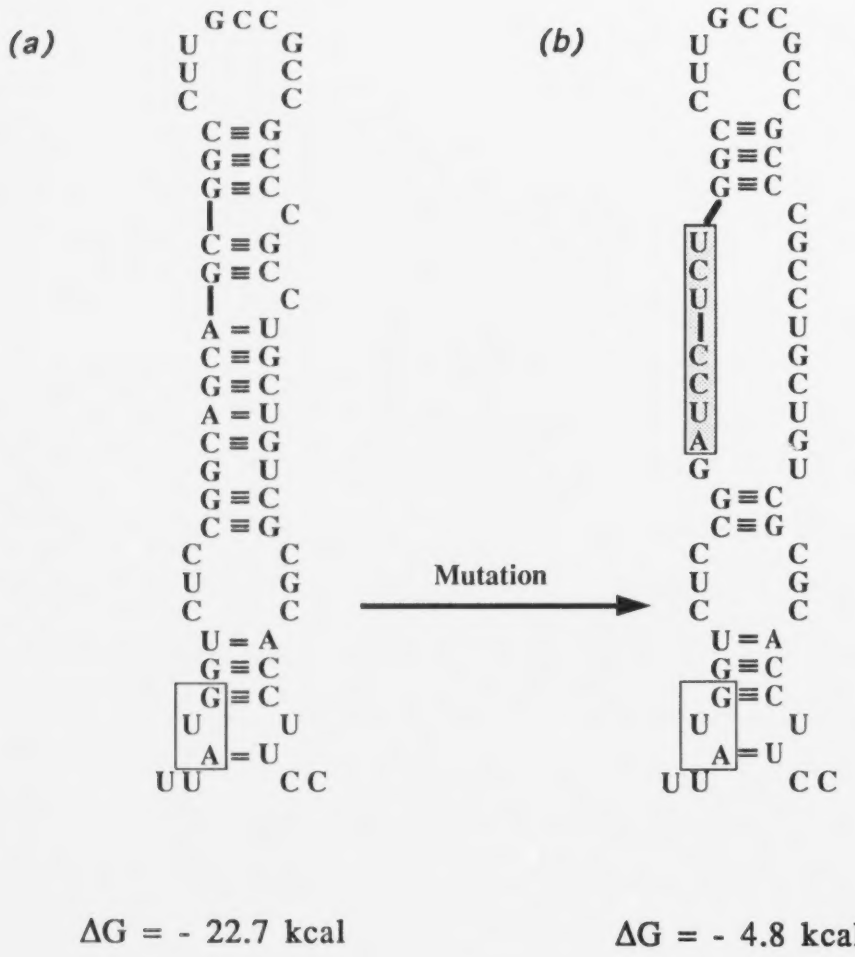


Figure 3.7. Prediction of mRNA secondary structure. The program of Zuker & Stiegler [36] was used to predict potential secondary structures in the mRNA from λSCS19-1. ***(a)*** Representation of the strong stem loop region found at the very start of the coding sequence. The AUG start codon found at the base of the stem is boxed as indicated. ***(b)*** Illustration of the predicted disruption in the secondary structure following mutation of six residues (shaded box) with a resultant decrease in free energy.

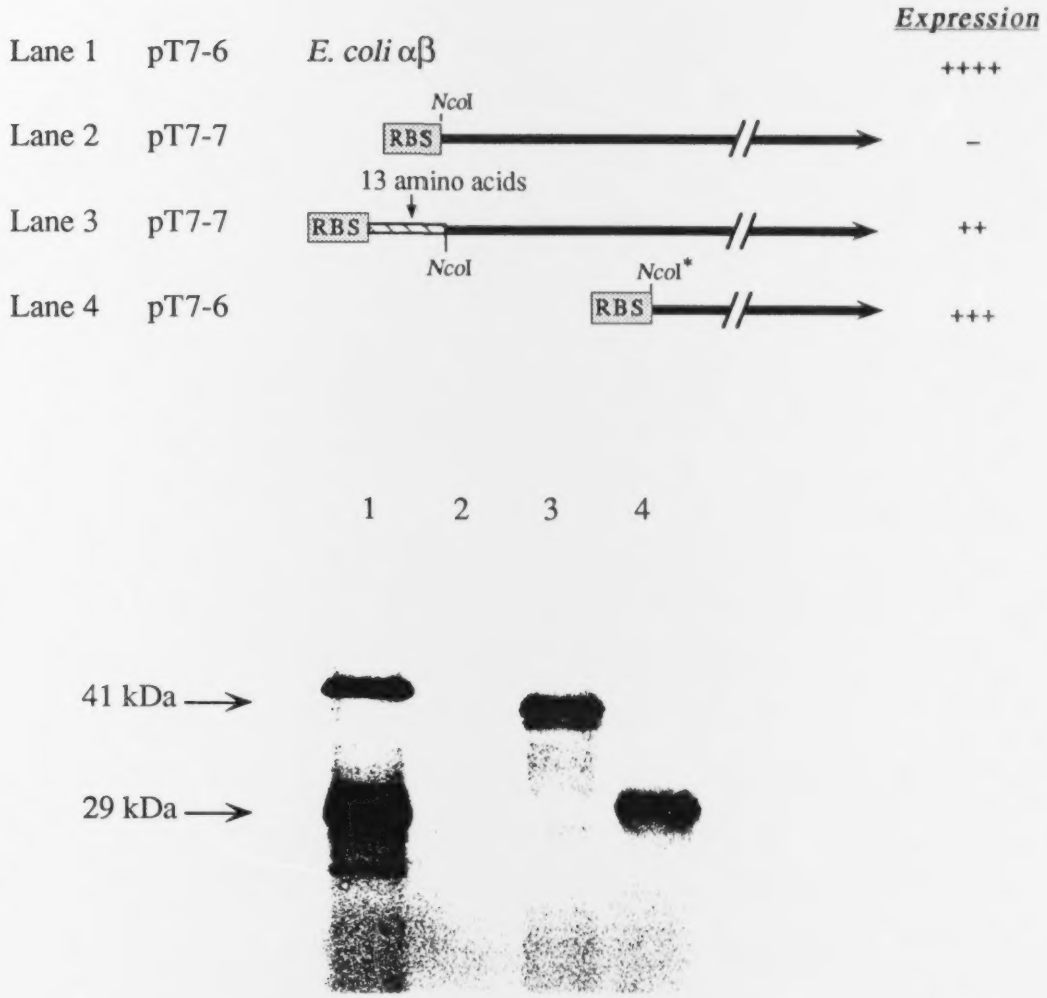


Figure 3.8. The effect of a leader sequence on the translation of λSCS19-1. Above is an autoradiograph of cell lysates resolved by SDS-PAGE. Analysis of expression of the complete open reading of λSCS19-1 placed at varying distances from the RBS site. Lane 2 represents the sequence placed immediately downstream of the RBS region of pT7-7. Lane 3 shows the effect of insertion of sequence encoding 13 amino acids upstream from the 5'end of the sequence. The positive control in lane 4 is pT7-6 containing the truncated λSCS19-1 sequence from *Nco*I* to *Eco*RI.

Expression of the pT7-7 recombinant carrying the 39 nucleotide spacer produced a labeled precursor protein (Fig. 3.8, lane 3), whereas the pT7-7 construct lacking the leader sequence remained blocked in its expression (Fig. 3.8, lane 2). This supports the suggestion that the proximity of λSCS19-1 sequences to the TIR is responsible for the failures in expression. It has been possible to reduce the length of the spacer to just nine nucleotides without adversely affecting the production of the precursor protein.

IV. DISCUSSION

The precursor form of the rat liver α-subunit failed to accumulate in bacterial cells, while the mature form was produced at levels similar to those seen with the bacterial enzyme. The sequence encoding the N-terminal signal peptide was found to be responsible for the lack of expression. Initiation of translation was found to be adversely affected by the proximity of the 5' end of the sequence to the TIR. Insertion of a leader sequence between the ATG initiator and the start of the cDNA reading frame allowed efficient production of a precursor form of the subunit. The need for the 5' end of the sequence to be in close proximity to the TIR suggests that intramolecular base-pairing interactions are involved.

There is a point of wide applicability to be made here, namely that for effective expression in *E. coli* the sequence at the extreme 5' end of foreign genes must be optimized so as not to interfere with the structure of the translation initiation region. One effective way of dealing with this problem is to make use of vectors that express

foreign genes as fusion proteins, joined in frame to the C-terminus of a highly expressed bacterial gene. It has been widely held that the use of such systems is advantageous because it offers a level of stability and protection from degradation to the foreign protein. One must also recognize an additional advantage to this strategy, possible interference with translation initiation is obviated.

The additional sequence at the N-terminus of the precursor has been reduced to Met-Ala-Arg. Since these residues are normally found in abundance in signal peptides, their presence at the N-terminus of a precursor would not be expected to effect its function. The precursor will, therefore, be useful in carrying out studies on the mitochondrial translocation of the subunit. Furthermore, the successful production of the mature form of the subunit will facilitate future studies on assembly of the mammalian enzyme.

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Chapter 4

Folding and assembly of the α-subunit are compromised in the presence of a signal peptide

I. INTRODUCTION

Subunits of mammalian succinyl-CoA synthetase are synthesized in the cytosol of the cell as higher molecular weight species called precursors [1,2]. These precursors contain N-terminal signal sequences that serve to direct the translocation of the subunits across the mitochondrial membranes. The signal sequences are removed during the translocation process, releasing the mature subunits for assembly in the matrix of the mitochondrion [3].

Several studies of protein translocation have revealed that at least a partial unfolding of the precursor is required for its transport. First, precursors trapped while translocating across the mitochondrial membranes showed portions of the protein exposed on both surfaces suggesting a vectorial unfolding as it passes through the membrane [4]. In the second study, folate analogs were found to block mitochondrial import of a dihydrofolate reductase precursor [5]. By binding to the protein, folate analogs prevent its unfolding. Furthermore, when the precursor is denatured by urea or destabilized by point mutations, its import is dramatically stimulated [6,7]. The translocation competence of a precursor was shown to correlate with the protein being loosely folded as judged by its protease-sensitivity [8]. Thus, it is now generally accepted that polypeptides requiring translocation across biological membranes must be presented to the translocation machinery in a loosely folded conformation.

Randall and Hardy have suggested that attainment of the translocation competent state is an intrinsic property of the

precursor molecule, imparted on it by the presence of the signal peptide [9]. Decelerated folding kinetics were observed for the precursors of maltose- and ribose-binding proteins [10] and for preβ-lactamase [11]. The precursors of several mitochondrial proteins have been found to adopt markedly different conformations from their mature counterparts [12,13]. Conformational differences have also been suggested in a number of cases where precursors form high molecular aggregates when synthesized *in vitro* [13-15]. Furthermore, the universal observation that signal sequences are removed from most translocated proteins suggests that their removal is a necessary prerequisite for folding and assembly to occur. In cases where the removal mechanism is blocked, cells become inviable [16]. Thus, it is possible that signal sequences may directly modulate the folding properties of their attached proteins to allow for translocation.

A cDNA encoding the precursor form of the α-subunit of rat liver succinyl-CoA synthetase has been expressed in bacteria (Chapter 3). This precursor provides a suitable model with which to address questions of protein conformation. The enzyme becomes phosphorylated on a histidine residue within the α-subunit during catalysis [17,18]. The α-subunit is capable of carrying out this phosphorylation in the absence of the β-subunit [19]. Thus, the lone α-subunit can form a conformation capable of both binding and hydrolyzing nucleoside triphosphate (NTP). Using procedures described in Chapter 2, it should be possible to produce, purify and carry out folding studies on the α-subunit precursor.

II. MATERIALS AND METHODS

A. Bacterial strains, plasmids and reagents

The following strains of *E. coli* were used in this study: JM109 was employed for molecular cloning; uracil-enriched DNA used for mutagenesis was generated in CJ236; expression was carried out in BL21(DE3). The genotypes of these strains can be found in **Appendix I**. The plasmids, pT7-6 and pT7-7, were provided by Dr. Stan Tabor of Harvard. Restriction endonucleases and additional DNA modifying enzymes were purchased from Bethesda Research Laboratories and New England Biolabs. Chemicals and reagents were bought from Sigma or British Drug House. Radioactive materials were supplied by Amersham Canada.

B. Plasmid construction

The construction of plasmids used in this study is illustrated schematically in Figures 4.1 and 4.2. The plasmids, pGSα and pGSβ, have been described in chapter 2. The sequence encoding the mature rat liver α-subunit was cloned in pJLA Rlα. These three gene constructs were transferred to the T7 vector, pT7-6 as illustrated in Figure 4.1. The plasmid, pT7 pRlα, was generated for the expression of the precursor form of the rat liver α-subunit (Chapter 3). This plasmid was modified by replacing the sequence encoding the mature subunit with that of the *E. coli* α-subunit, creating pT7 pEcα (Fig. 4.2). All genetic manipulations were performed according to standard procedures [20].

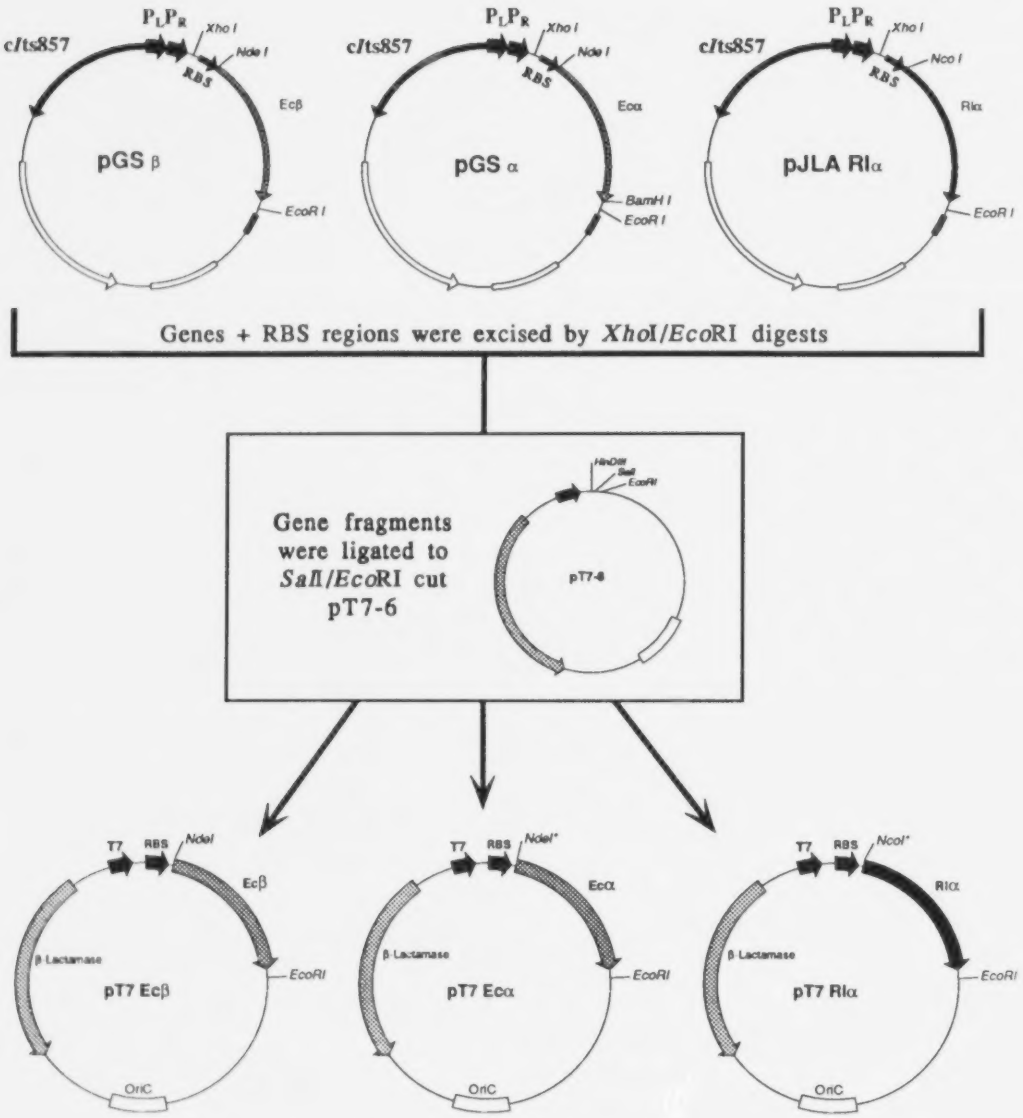

Figure 4.1. Schematic illustrating the construction of expression plasmids for the mature subunits of the enzyme. The genes encoding the E.coli α- and β-subunits (designated Ecα and Ecβ) and the cDNA sequence encoding the mature rat liver α-subunit (designated Rlα) were removed from the control of the $\lambda P_L P_R$ promoter in the pGS and pJLA vectors and placed under the control of a T7*ϕ10* promoter in the plasmid, pT7-6.

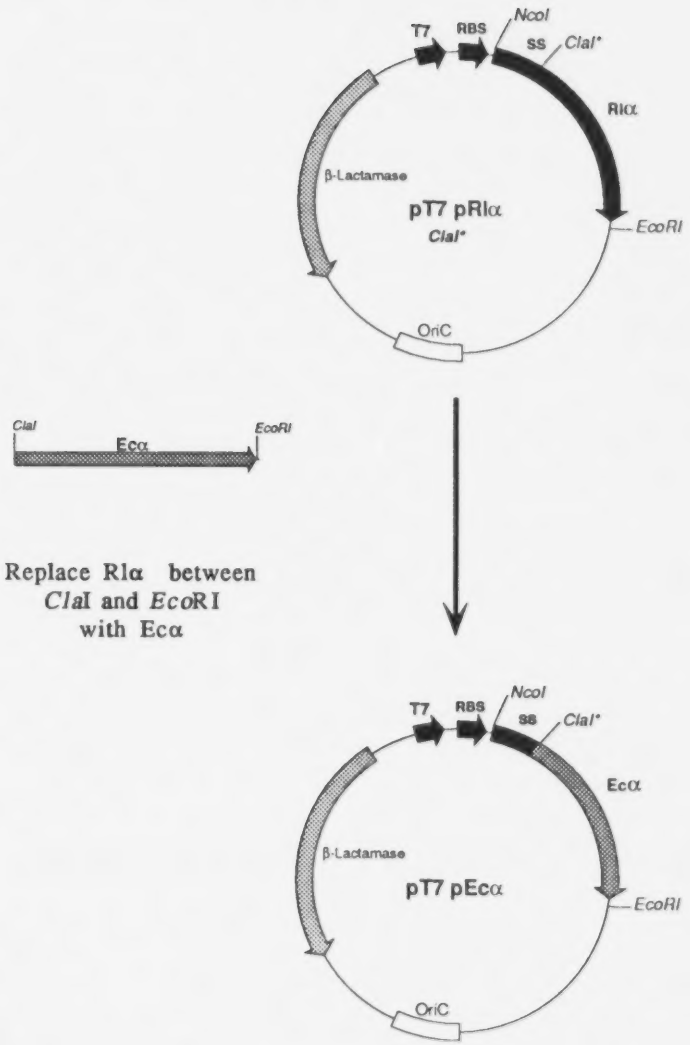

Figure 4.2. Schematic illustrating the construction of expression plasmids for the precursor forms of the α-subunit. The cDNA for the precursor of the rat liver α-subunit (designated pRlα) has previously been cloned in pT7-7. The sequence encoding the mature portion of the precursor molecule was replaced as shown above.

C. Expression and purification of subunits

Cultures of BL21(DE3) cells containing the pT7 recombinants were grown at 37 °C in LB broth supplemented with ampicillin (50 μg/ml). See **Appendix III** for medium composition. When the A_{600} reached ~0.6, expression was induced by the addition of IPTG to a final concentration of 0.5 mM. Cells were harvested by centrifugation after 5 hours. Cell pellets were resuspended in 0.2 volume (relative to culture volume) of ice cold sonication buffer: 100 mM Tris HCl (pH 7.4), 100 mM potassium phosphate, 100 mM KCl, 1 mM EDTA. Suspensions were sonicated and the resulting lysates were centrifuged at 15,000x g for 5 minutes. Supernatants were discarded and the pellets were washed in a further 0.2 volume of sonication buffer. The suspensions were then centrifuged at 100x g and the pellets were solubilized in 0.1 volume of denaturing buffer: 6.0 M GuCl, 50 mM Tris HCl (pH 7.4), 0.1 mM EDTA, 0.1% β-mercaptoethanol. Solubilized protein pellets were left standing at room temperature overnight to effect complete denaturation. The samples were clarified by centrifugation at 15,000x g for 30 minutes to remove any cell debris.

D. Refolding and autophosphorylation

The denatured subunits were refolded using a modification of the method of Pearson and Bridger [21]. Equimolar mixtures of the subunits were diluted into refolding buffer (100 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, 0.5 mM ATP, 5 mM DTT) such that the final concentration of denaturant (GuCl) was reduced to 0.2 M or less. Protein concentrations in the refolding mixtures were

in the range of 50-100 µg/ml. Refolding was allowed to proceed for 1 hr at 20 °C after which samples were assayed for enzymatic activity using the spectrophotometric method [22]. Autophosphorylation was carried out according to the method of Pearson and Bridger [19]. Denatured α-subunit was diluted 25-fold into phosphorylation buffer (50 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (~50 µCi/µmol), 5 mM DTT). The reactions were left at room temperature for 24 hr. Samples were immunoprecipitated, resolved on SDS-PAGE gels, and subjected to autoradiography.

E. Labeling and pulse- chase

Procedures for exclusive labeling of plasmid-encoded proteins using the T7 RNA polymerase/promoter system were modified from those described by Tabor & Richardson [23]. Cultures of BL21(DE3) cells containing the respective expression vectors were grown to an A_{600} of ~0.5. One ml of culture was withdrawn and centrifuged. The cell pellet was washed twice in 1 ml of M9 medium (**Appendix III**) prewarmed to 37 °C. The final cell pellet was resuspended in 1 ml of M9 medium supplemented with the following: 0.02 % casamino acids depleted of methionine (Gibco), 0.05 % glucose and 0.5 µg/ml thiamine. Cultures were incubated at 37° C for 1 hr and then induced with IPTG. Expression was allowed to proceed for 15 minutes after which the cultures were treated with rifampicin at a final concentration of 400 µg/ml. Cultures were left standing at 37° C for 45 minutes before the addition of 10 µCi of [^{35}S]-methionine/cysteine label (Trans-label, ICN). After 10 minutes

of labeling, 20 µl of a 1 % methionine solution was added. Aliqouts of 200 µl were removed at 0, 15, 30, 45, and 60 minute intervals following the chase. These aliquots were immediately chilled in ice water, centrifuged at 15,000x g for 2 min and the cell pellets were solubilized in 100 µl of SDS-PAGE sample loading buffer. The denatured proteins were resolved on 11 % SDS-PAGE, dried under vacuum and subjected to autoradiography.

III. RESULTS

A. PRECURSOR FORMS OF THE α-SUBUNIT ARE UNSTABLE *IN VIVO*

Plasmids capable of producing mature and precursor forms of both the rat liver and *E. coli* α-subunits were created in pT7-6 (Fig. 4.1 and 4.2). The precursor form of the *E. coli* subunit was generated by fusion of the mature subunit to the signal sequence of the rat liver precursor. Although this *E. coli* precursor does not occur in nature, its inclusion in this study will provide comparison for observations made with the mammalian precursor.

Cultures of BL21(DE3) cells containing pT7 recombinants were grown up and induced as outlined in **Materials and Methods**. Samples were removed at various times following induction and analyzed for the amounts of accumulated subunit (Fig. 4.3). Differences were clearly seen between the levels of accumulation of the precursor and mature forms of the subunits. The precursor forms of both subunits were found to accumulate at considerably lower levels than their corresponding mature forms. In addition, the two

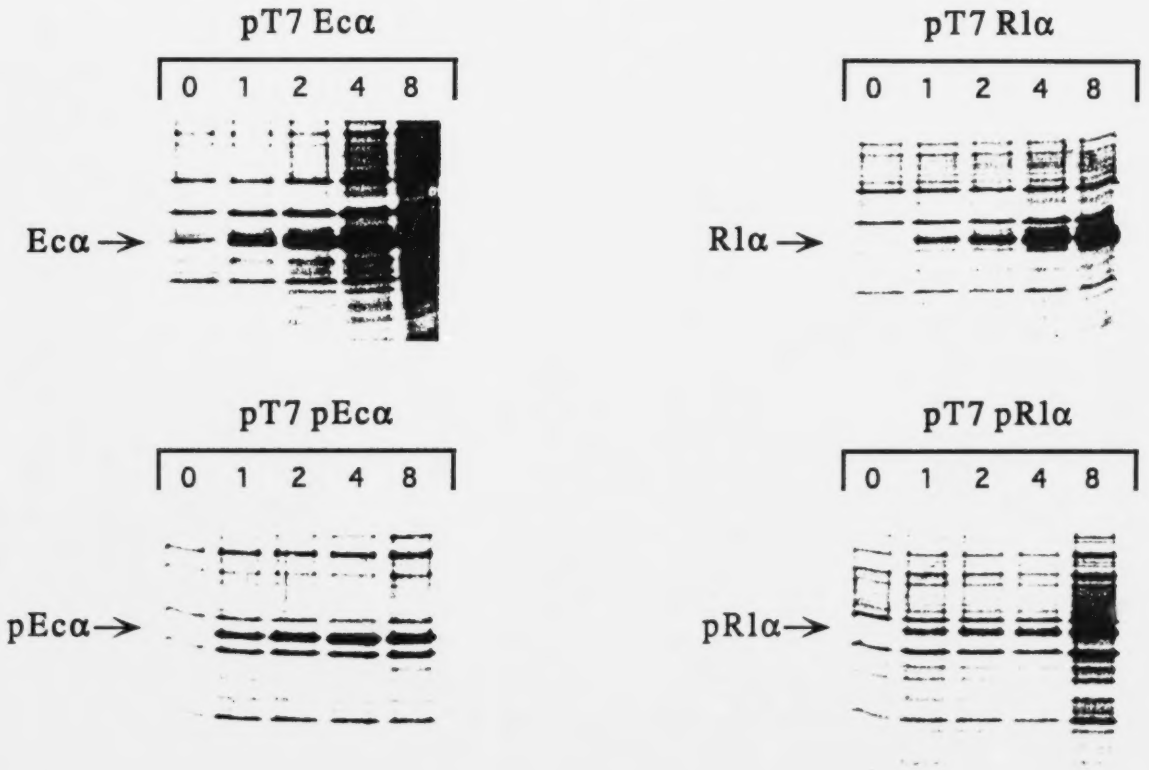

Figure 4.3. Production of mature and precursor forms of the α-subunit of pig heart and *E. coli* succinyl-CoA synthetase. BL21(DE3) cells containing the expression plasmids, pT7 Ecα, pT7 Rlα, pT7 pEcα and pT7 pRlα, were grown in LB broth to an A_{600} of 0.5. Expression was induced by the addition of IPTG. See **Materials and Methods** for details. Samples were withdrawn from the cultures at 0, 1, 2, 4 and 8 hours following the induction, centrifuged and the cell pellets were lysed in SDS-PAGE running buffer. Samples were analyzed by SDS-PAGE followed by Coomassie staining.

forms of the mammalian subunit were found in lower levels compared to the two forms of the *E. coli* subunit.

Cultures producing both forms of the two subunits were subjected to short pulses of [^{35}S]methionine labeling. Similar amountss of label incorporation were found between the precursor forms and their corresponding mature forms. Thus, the lower levels of accumulation of the precursor proteins do not result from lower rates of synthesis. Slightly lower rates of synthesis were detected for the two mammalian forms compared to the *E. coli* forms. These lower rates are reflected in the lower levels of accumulation seen with the mammalian subunit. This may be due to less optimal codon usage in the reading frame of the mammalian sequence. Pulse-labeled cultures were chased for varying lengths of time with excess unlabeled methionine. Marked differences were seen in the rates of disappearance of labeled subunits (Fig. 4.4). The precursor form of both the *E. coli* and the rat liver subunits was degraded at a much faster rate than its corresponding mature form. The difference was estimated, over a series of experiments, to be tenfold in magnitude. Thus, the lower levels of accumulation seen with the precursors result from a higher rate of breakdown in the cell.

These results can be interpreted in terms of the different conformations the two forms of the subunit might adopt. The mature α-subunit would be expected to form a native-like conformation consistent with its ability to autophosphorylate [19]. This would likely be a tightly folded structure, one that shows some resistance to proteolysis. The higher breakdown rate seen with the precursor is suggestive of it having a looser conformation. This interpretation is

Figure 4.4. The stability of subunits in bacterial cells. Cultures of BL21(DE3) cells carrying the expression plasmid for two forms of the *E.coli* and the rat liver α-subunits were grown to an A_{600} of 0.5. Cells were labeled with [^{35}S] methionine for 10 minutes after which unlabeled methionine was added in 1,000 fold excess. Aliquots were removed at 0, 15, 30, 45 and 60 minutes following this addition and immediately dissolved in SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE.

consistent with reports that precursors tend to adopt structures that are protease-sensitive to translocate [8,9]. It is, however, also a possibility that the signal sequences of the precursors make these proteins better targets for N-terminal proteases. The two forms of the *E. coli* subunit behaved similarly to the rat liver subunit. Thus, analogies can be made between the bacterial and mammalian subunits.

B. The precursor forms are unable to phosphorylate

Both the precursor and mature forms of the two subunits were found to accumulate as inclusion bodies in *E. coli* cells. The subunits were purified from cell lysates as described in **Materials and Methods**. The purified subunits were tested for their abilities to phosphorylate. If the conformation of the precursor is distinctly different from that found in the mature subunit, then, we would expect the precursor to be compromised in its ability to phosphorylate.

The two forms of each of the subunits were renatured in phosphorylation buffer and allowed to phosphorylate over a period of 24 hours. Samples of each of the reactions were subjected to immunoprecipitation and the proteins were resolved on SDS-PAGE and subjected to autoradiography (Fig. 4.5*a*). Absolutely no phosphorylation was seen with the precursors whereas the mature subunits displayed their usual capacity to phosphorylate. These results suggest that the precursor does not form a conformation necessary for autophosphorylation. The reactions were carried out over an extended period of time and yet no traces of radioactive

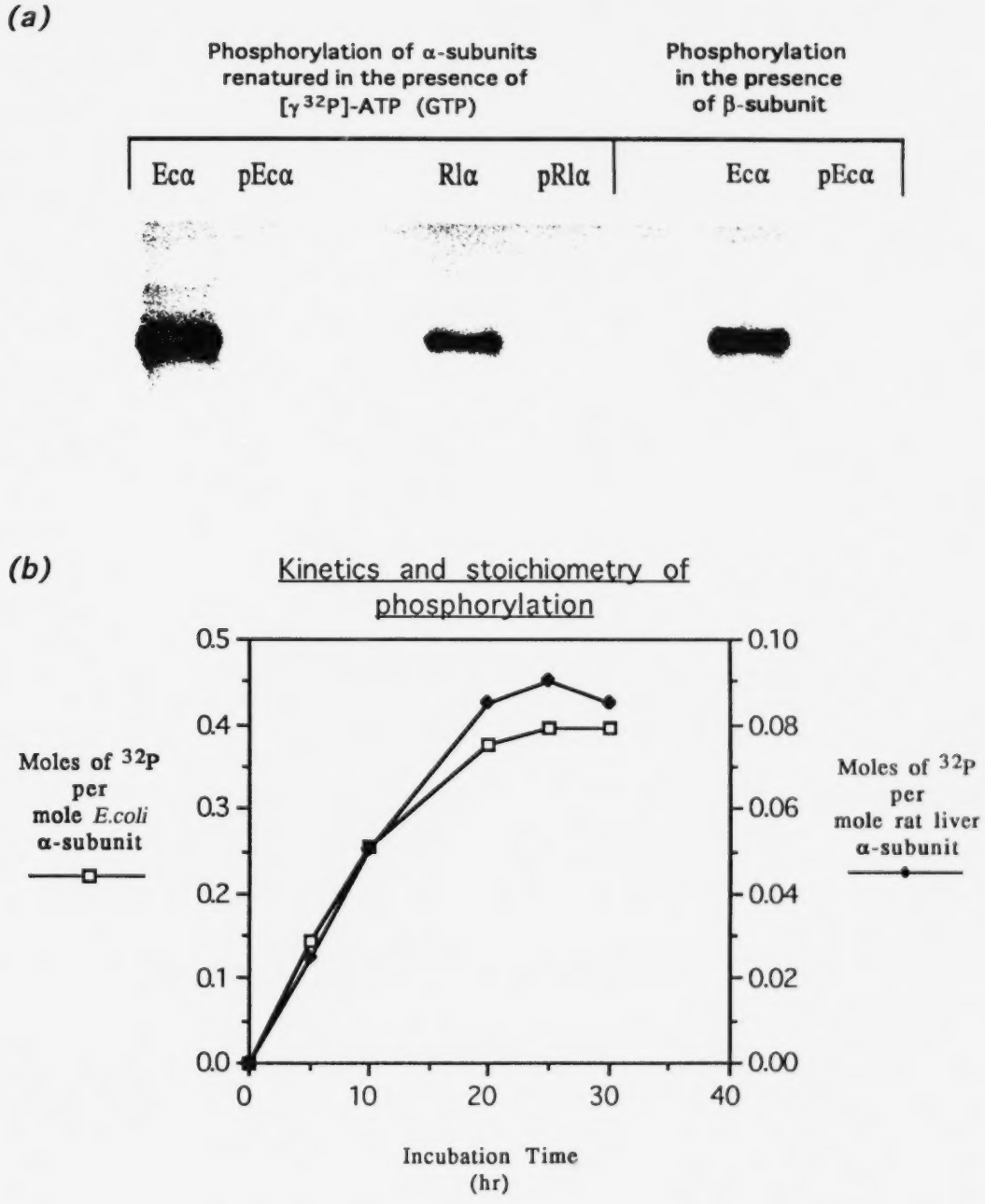

Figure 4.5. Phosphorylation of the two forms of the α-subunit. Denatured α-subunits were diluted out in a refolding buffer containing [^{32}P]γATP. GTP was used in the case of the rat liver forms. The final concentration of denaturant was below 0.2 M and the amounts of protein were equivalent (55 μg/ml). ***(a)*** Phosphorylation levels in the subunits were assessed after 24 hours by immunoprecipitating the proteins and resolving them on SDS-PAGE. ***(b)*** Aliquots were removed from the reactions after varying lengths of time. The protein was separated from free radioactive label by phenol extraction. The phenol phase was then counted by liquid scintillation.

label could be found in the precursors. It is, therefore, unlikely that the signal sequence is modulating the folding of the subunit in a temporal manner as was reported for other precursors [10,11]. An alternative interpretation of this result would suggest that the signal sequence interferes with the binding of the nucleotide, thus, preventing phosphorylation from taking place. However, such steric hindrance would not explain the increased protease-sensitivity of the precursors *in vivo*.

The degree of phosphorylation seen with the mammalian subunit was consistently lower than that seen with the *E. coli* subunit despite the presence of equivalent amounts of protein. The kinetics and stoichiometry of phosphorylation of the two subunits were determined (Fig. 4.5*b*). Both subunits were found to have similar rates of phosphorylation, reaching their maximum phosphorylation states by 25 hours. The *E. coli* protein contained 0.37 moles of ^{32}P per mole of subunit when fully phosphorylated. The degree of phosphorylation of the subunit (37 %) correlates well with its ability to reconstitute enzyme activity (40 %, Chapter 2). In fact, the mature *E. coli* subunit showed similar levels of phosphorylation when renatured in the presence of the β-subunit (Fig. 4.5*a*). In this context, it is not surprising to find that the maximum level of phosphorylation achieved by the rat liver subunit is around 10 %. Previously reported values for the reconstitution of the mammalian enzyme from its denatured subunits are similarly low [24]. Thus, it appears that the phosphorylation capacity of the two subunits correlates with their ability to reconstitute active enzyme.

C. THE PRECURSOR FAILS TO ASSEMBLE WITH THE β-SUBUNIT

Since the phosphorylation capacity of the subunits correlates with the ability of subunits to fold and assemble, the precursors would not be expected to assemble. The mammalian β-subunit was not available at the time this study was undertaken. Therefore, reconstitution tests were only carried out with the *E. coli* subunits. However, the results seen so far with the *E. coli* precursor parallel those observed for the mammalian precursor and are thus relevant to the overall study.

Repeated attempts at refolding the precursor form of the *E. coli* α-subunit with its companion β-subunit failed to produce any detectable enzyme activity above controls (Table 4.I). It was possible that the precursor folded and assembled with its companion β-subunit, but, due to the presence of the signal sequence, the assembled product was inactive. Reconstitution reactions were carried out with the mature α- and β-subunits both in the presence and absence of an equivalent amount of the precursor form of the α-subunit. If the precursor was folding correctly and interacting with the β-subunit, we should see a competition between the two forms of the α-subunit for assembly with the β-subunit. In such case, lower reconstitution activities would be expected in the presence of the precursor. However, no difference was seen in the levels of reconstituted activity when the precursor was present (Table 4.I). In one set of reactions the precursor was present at tenfold higher levels than the mature subunits and still had little effect on the final outcome. It appears as though the precursor is unable to interact with the β-subunit in any manner.

Table 4.I

Refolding and assembly of the two forms of the α-subunit

Subunit composition of refold[1]	Stoichiometry of subunits	Reconstituted activity (units/ml)		
		Expt.I	Expt.II	Expt.III
α		0.002		
β + α	1:1	0.475	0.53	0.50
pα		0.0015		
β + pα	1:1	0.0018	0.0025	0.002
β + α + pα	1:1:1	0.48	0.46	0.49
β + α + pα	1:1:10		0.445	0.49

Equimolar mixtures of the α-, β- and precursor (pα) α-subunits (except where pα was present in tenfold excess) were diluted into refolding buffer: 100 mM potassium phosphate (pH 7.4), 10 mM $MgCl_2$, 0.5 mM ATP, 5 mM DTT, such that the final concentration of denaturant (GuCl) was reduced to 0.2 M. The protein concentrations in the reconstitution mixtures were 65 μg/ml. Refolding was allowed to proceed for 1 hr at 20 °C after which samples were assayed for enzymatic activity.

IV. DISCUSSION

In conclusion, the precursor is compromised in its abilities to fold, phosphorylate and assemble into an enzyme complex. The presence of a mitochondrial signal sequence at the N-terminus of the precursor appears to prevent the attached subunit from forming the native-like conformation. Although alternative reasons have been given to explain each of the results, the data as a whole fits well with the conformational argument. The increased sensitivity of the precursor to degradation *in vivo* is suggestive of it forming a translocation competent conformation in line with previous reports correlating the translocation competence of precursors with the protein having a loosely folded, protease-sensitive structure [8]. Furthermore, the inability of the precursor to carry out its own phosphorylation or to interact with a mature β-subunit suggests the absence of a native-like conformation in the precursor. These findings are very much in harmony with reports in which precursors of several other mitochondrial proteins were found to adopt markedly different conformations from their mature counterparts [12,13].

Findings that the presence of a mitochondrial signal sequence in precursor forms of the α-subunit alters the conformation that these subunits adopt are significant to the study of protein translocation. As mentioned earlier, it has been suggested that the attainment of a translocation competent state is an intrinsic property of precursor molecules [9]. Over time, proteins have evolved a set of commands within their primary sequences to accurately fold into

their native structures. In view of the precision required it is not difficult to imagine how the process could be so sensitive to additional folding information as is present in the signal sequence. A model in which the signal sequence is responsible for the attainment of the translocation competent state of a precursor is both elegant and simple. In particular, its covalent attachment to the protein may be a crucial factor given the rapidity of the folding process. Achieving the competent state is made intrinsic to the precursor and does not depend on external factors.

Recent investigations have implicated a plethora of cellular proteins in retrieving and maintaining the translocation competent state of precursor proteins [25,26]. However, under normal physiological conditions, such factors may not be necessary since in most cases the transport of precursors is a rapid process that does not require the precursor to be maintained in an unfolded state for long [27-30]. Furthermore, most precursors are cleared from the cytosol relatively fast indicating that the cell does not tend to maintain such precursors for extended periods of time [27].

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Section III

Mammalian Isoforms

Chapter 5

Alternative mRNA Splicing Produces a Novel Isoform of Pig Heart Succinyl-CoA Synthetase

I. INTRODUCTION

Succinyl-CoA synthetase from all mammalian sources examined to date is an $\alpha\beta$ dimer [1-3]. In contrast, the enzyme from *E. coli* [4-6] and a variety of other gram-negative species [7-9] is an $\alpha_2\beta_2$ tetramer. The three-dimensional structure of the tetrameric *E. coli* enzyme has been determined recently [10]. This represents a significant advancement in our understanding of the enzyme, particularly with respect to its catalytic mechanism and quaternary structure. The elucidation of a similar structure for the mammalian dimeric enzyme would be of interest. Such information would help us to understanding the significance changes in the quaternary structure have for the function of this enzyme.

It was reported that the mammalian enzyme exists in various forms. Baccanari and Cha [11] showed that the enzyme from pig hearts separated into several species differing in charge. These multiple forms were, however, found to be inter-convertible indicating a common protein origin. In later studies, Weitzman et al. [12] demonstrated the existence of isoforms of succinyl-CoA synthetase in mammalian tissue that differed in their nucleotide specificity. Separable GTP- and ATP-specific forms were present in ratios that varied depending on the tissue source. Increases were seen in the levels of a GTP-specific form in brain tissue following treatment of rats with streptozotocin, a drug used to induce diabetes [13]. In addition, a distinct GTP-specific form of the enzyme was induced in mouse liver during increased porphyrin synthesis [14]. Interestingly, although isoforms of the bacterial enzyme have not

been reported, the enzyme from *E. coli* exhibits less stringent specificity in its use of nucleotides, accepting either ATP or GTP, the former being preferred [15].

The reports of polymorphism in the pig heart enzyme precluded it from being selected for crystallographic studies. Obtaining isomorphically pure preparations of an enzyme is a necessary prerequisite to crystallographic work. As a step in this direction, a cDNA library has been screened for sequences encoding the α-subunit of the pig heart enzyme. These sequences can be expressed in *E. coli* in combination with that of the β-subunit [16] to produce an enzyme suitable for both structural and biochemical characterization. The following report describes the isolation of cDNAs encoding two forms of the α-subunit of pig heart succinyl-CoA synthetase.

II. MATERIALS AND METHODS

A. Bacterial strains, enzymes and reagents

The following *E. coli* strains were used in this study: JM109 was routinely used for the construction and propagation of M13 and plasmid derivatives, screening of cDNA and genomic libraries was performed in Y1089 and LE392, respectively. The genotypes of these strains can be found in **Appendix I**. Kinase, ligase, Klenow DNA polymerase and restriction enzymes were supplied by Bethesda Research Laboratories and New England Biolabs. *Taq* DNA polymerase was purchased from Cetus Corp. Reagents for sequencing were from United States Biochemicals. dNTPs were purchased from

Pharmacia as 0.1 M solutions. All other chemicals were supplied by Sigma and British Drug House.

B. cDNA LIBRARY SCREENING

The generation of the pig heart cDNA library used in this study has been reported elsewhere [17]. The unamplified library was screened using a probe (labeled with biotin-11-UTP, Bethesda Research Labs. Inc.) derived from the cDNA insert of λSCS19-1. This clone encodes the precursor to the α-subunit of rat liver succinyl-CoA synthetase [18]. Clones were identified using the screening procedures of Huynh *et al.* [19]. Development of the biotin based probe was achieved using streptavidin conjugated alkaline phosphatase according to the methods of Haas and Fleming [20]. The cDNA inserts of isolated clones were subcloned into M13 and sequences were determined by the dideoxynucleotide chain termination method [21].

C. GENOMIC DNA LIBRARIES

High molecular weight DNA was prepared from the liver of a mature pig by standard methods [22]. The DNA obtained had an average molecular size greater than 50 kb. This was used both to construct a library and for direct PCR reactions. A library was made in λGEM11 according to standard procedures [23]. λGEM11 arms and packaging extracts were supplied by Promega. A second pig genomic library in λEMBL-3 was purchased from Clontech Inc.. Both libraries were screened with the PHα57 clone which was random-prime labeled with [^{35}S]α-dATP according to the methods of Feinberg and

Vogelstein [24]. Approximately 1.0×10^6 plaques were screened from both libraries using high stringency hybridization procedures.

D. Southern hybridization

DNA from the genomic clones was subjected to the appropriate restriction enzyme digestions. The resulting fragments were resolved on 0.8 % agarose gels and transferred to GeneScreen membranes (DuPont) using a wet transfer blotting apparatus (BioRad). Membranes were probed according to the established procedures. Oligonucleotides were end-labeled with [^{32}P]ATP and T4 DNA kinase. Temperatures for oligonucleotide hybridizations were 5 °C lower than their respective T_m.

E. Polymerase chain reaction (PCR)

PCR was carried out according to the methods of Saiki et al. [25]. The sequences of the oligonucleotides used can be found in **Appendix II** #5 through to #10. These oligonucleotides were also used as hybridization probes in Southern and Northern blots. The cDNA sequence to which these oligonucleotides hybridize is shown in Figure 5.3. Oligonucleotides #7, #9, and #10 contained the restriction sites *Hin*DIII, *Bal*I, and *Sph*I, respectively, to aid in cloning of PCR generated fragments. Amplification products were purified by electrophoresis through 1.2 % agarose (BRL) and were extracted from the gels using the glass milk method [26]. Purified fragments were digested with restriction enzymes where necessary and cloned into M13 for sequence determination.

III. RESULTS

A. ISOLATION OF cDNA CLONES ENCODING THE α-SUBUNIT

Aproximately 1.0×10^4 plaques from an unamplified pig heart cDNA library were probed with biotin-labeled cDNA encoding the rat liver α-subunit. A total of 25 clones were isolated from three separate hybridization screenings. As shown in Figure 5.1, the clones were categorized on the basis of size into three groups as follows: Group I contained thirteen of these clones; all of which were composed of a single 900 bp *Eco*RI insert. The remainder of the clones were larger in size and produced two insert fragments from *Eco*RI digestion, indicating an internal *Eco*RI site. These clones could be further divided into two groups on the basis of the larger *Eco*R I fragment. Nine of them contained a 1.15 kb *Eco*RI fragment and were designated Group II. The three remaining clones, classified as Group III, had a slightly larger (1.2 kb) fragment. The smaller *Eco*R I fragment of the latter two groups varied in length from 50 to 120 bp, suggesting that this may represent the 5' end of the message.

Representatives from each group was selected for sequencing. The longest from group II and III with respect to their smaller *Eco*R I fragment were selected. When aligned to each other the three clones were found to be identical throughout their sequence with the exception of one short region midway through the open reading frame. The 3' untranslated region was similar in length (220 residues) in all cases followed by a short polyA tail 15-20 nucleotides long. The smaller *Eco*RI fragment did indeed represent

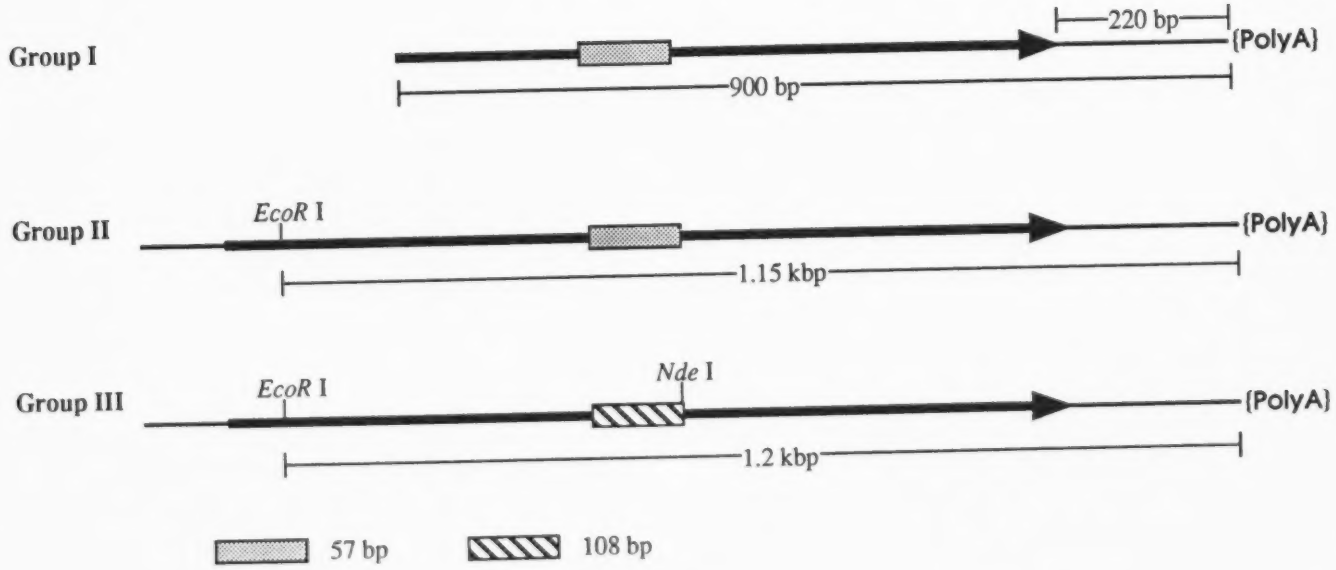


Figure 5.1. Alignment of the three groups of cDNAs for the α-subunit. Representative isolates from each of the three groups of cDNAs are aligned on the basis of sequence identity. Arrows indicate the open reading frames. Shaded boxes represent regions of sequence divergence. A unique *Nde*I site is shown above the shaded box of the Group III clone. The sizes of the divergent sequences are indicated below.

the 5' end of the message. The longest sequence contained just 42 nucleotides of the untranslated sequence. The complete 5' end has yet to be determined (see Discussion).

The nucleotide and deduced amino acid sequences are shown in Figure 5.2*a* and *b*. The complete sequence of a Group II clone, hereinafter referred to as PHα57, is given in Figure 5.2*a*. This sequence contained an open reading frame encoding a 333 amino acid residue protein. The sequence of the clone from Group I was identical to the equivalent portion of PHα57. The 5' end of this clone terminated at a C residue immediately preceding the Ile_{105} codon of PHα57. The amino acid sequence encoded by PHα57 exhibits an exceptionally high degree of identity to the precursor of the rat liver subunit [18], with 305 of the 333 residues identical. Both mammalian proteins carry a 27 amino acid signal peptide which is removed upon entry into the mitochondria [27]. The N-terminal sequence of the mature α-subunit was determined by dansyl-Edman degradation, confirming the location of the signal sequence cleavage site.

The sequence derived from the Group III clone, PHα108, was virtually identical to PHα57 except for a short region of coding sequence midway through the open reading frame. A stretch of sequence comprised of 57 nucleotide residues present in PHα57 was replaced by an entirely new sequence 108 residues in length (Fig. 5.2*b*). This new sequence is in frame and results in a novel 36 amino acid residue sequence that bears no resemblance to the corresponding region from PHα57. More importantly, this region of the PHα57 encoded protein is well conserved in the α-subunits of the

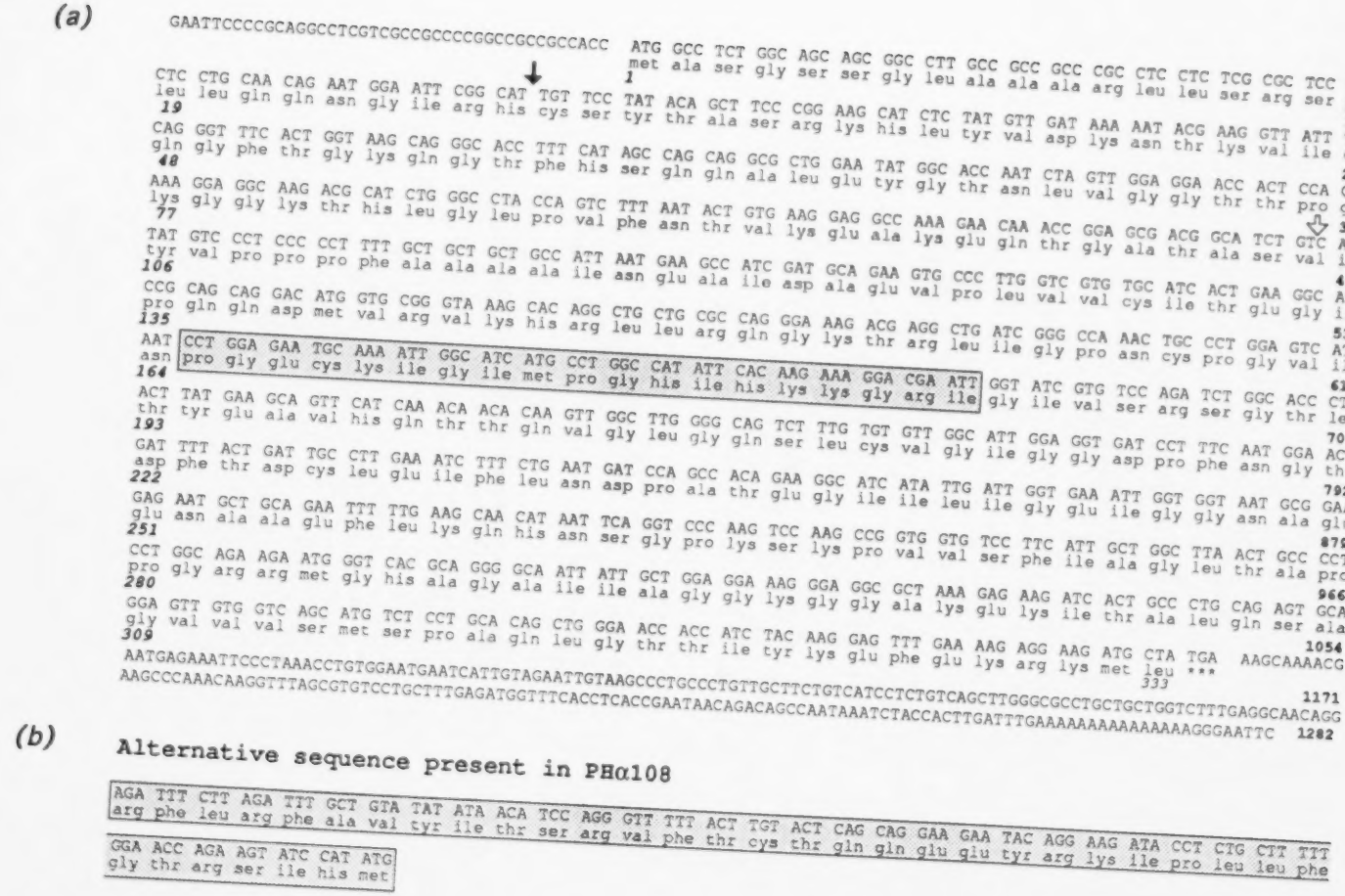


Figure 5.2. Sequences of cDNAs encoding the α-subunit of pig heart SCS. *(a)* The nucleotide sequence determined from PHα57 and its derived amino acid sequence. An open arrow marks the position of the 5' end of a Group I clone. A closed arrow shows the position of the signal sequence cleavage point. The shaded block indicates the region within the PHα57 sequence that is replaced by the alternative sequence *(b)* of PHα108.

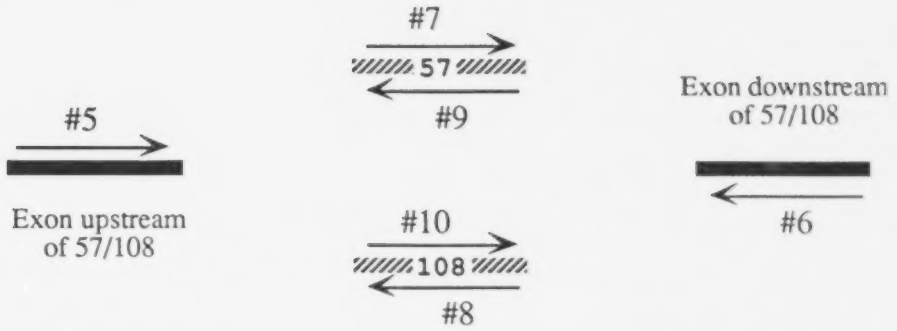
E. coli [28] *Thermus flavus* [29] and *Saccharomyces cerevisiae* [30] enzymes (see Discussion).

The identities of the remaining unsequenced clones, especially the two categorized as Group III, were confirmed. The presence of a unique *Nde*I site at the 3' end of the PHα108-specific sequence (see Fig. 5.1) was used as a discriminating characteristic. PCR tests using oligonucleotides specific to the two different sequences confirmed the identity of the remaining cDNAs. Furthermore, PHα108-specific sequences were amplified directly from the original cDNA library. These two different stretches of sequence shall be referred to as 57 and 108. Oligonucleotides complimentary to them will be called 57- and 108-specific, respectively.

B. Sequences unique to PHα108 are present in the gene

The protein encoded by PHα108 represents a novel alternative form of the α-subunit. Replacement of the more commonly encountered 57-encoded sequence by this 108-encoded sequence is highly suggestive of a mutually exclusive splicing event. If the two sequences represented alternative exons within a single gene, it should be possible to amplify, by PCR, that region of the gene carrying the two exons. To this end, a set of primers that were complementary to the cDNA sequences upstream and downstream of the 57/108 region were made (Fig. 5.3*a*, #5 and #6). Repeated attempts at amplifying a specific DNA fragment directly from genomic DNA using these primers failed. Nevertheless, further PCR reactions were performed using combinations of these two primers with either 57-specific (#7 and #9) or 108-specific (#8 and #10)

(a)



(b)

5'/3' Primer Pairs	Size of DNA
#5/#6	none detected
#5/#9	200 bp
#5/#8	none detected
#7/#6	none detected
#10/#6	1.3 Kbp
#7/#8	none detected
#9/#10	none detected

(c)

Figure 5.3. PCR amplification of the α-subunit gene from porcine DNA. ***(a)*** PCR was carried out using the primers indicated. The relative positions of the primers (#5-10) and the direction of their extensions are shown. ***(b)*** Fragments resulting from amplification with the respective primer pairs are listed. *None detected* indicates that no specific product was found to be amplified. Sequences of the primers are given in **Appendix II**. ***(c)*** Model showing the most likely position of the exons relative to each other and in keeping with PCR results. The sizes of two of the introns are shown.

oligonucleotides (Fig. 5.3*a*). Fragments of 200 bp and 1.3 kb were amplified in reactions involving the primer pairs, #5/#9 and #6/#10, respectively (Fig. 5.3*b*). All other combinations were unsuccessful in amplifying a specific product. A model of the exon arrangement in the α-subunit gene consistent with the above data is presented in Figure 5.3*c*. In this model the 57 exon is placed upstream of the 108 exon. The products of PCR reactions #5/#9 and #6/#10 represent the introns between the 57 exon and its upstream partner and between the 108 exon and its downstream partner. The model further proposes that the size of the region between exon 57 and exon 108 may be too large for conventional PCR amplification. This would explain why all other combinations of primers failed since they involve bridging the region between these exons.

C. Cloning the α-subunit gene

A pig genomic library constructed in λEMBL-3 was screened to obtain the region of sequence lying between the two exons. Using the PHα57 cDNA as a hybridization probe, two clones, designated as λE4 and λE6, were isolated and purified. Both of these clones were tested for their ability to hybridize to 57- and 108-specific oligonucleotides. The clones were subjected to southern blot analysis, the results of which are presented in Figure 5.4. Fragments of λE4 hybridized to the 108-specific probe but not the 57-specific one, whereas λE6 fragments hybridized only to the 57-specific probe. λE6 also hybridized to the oligonucleotide specific for the exon upstream of 57 (#5) and λE4 hybridized the oligonucleotide specific for the exon downstream of 108 (#6). In addition, we were able to reproduce the

Probing Genomic Clones for the presence of 57 and 108 Sequences

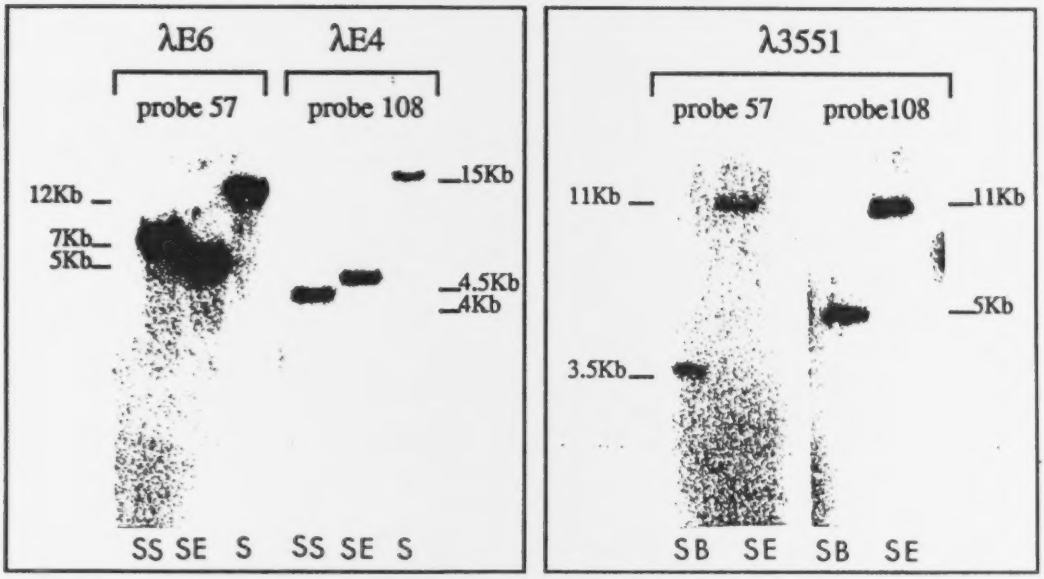


Figure 5.4. Hybridization of genomic clones to oligonucleotides specific for the alternative exons. DNA purified from the genomic clones, λE6, λE4 and λ3551 was digested with the following restriction enzymes: *Sal*I (S), *Sal*I/*Eco*RI (SE), *Sal*I/*Sst*I (SS) in the case of λE6 and λE4; *Sst*I/*Eco*RI (SE) and *Sst*I/*Bam*HI (SB) in the case of λ3551, resolved by electrophoresis and transferred to Genescreen membranes. The blots were probed as indicated by [^{32}P] labeled oligonucleotides specific for the 57 and 108 exons. The approximate size of each hybridizing fragment is indicated.

PCR amplifications using the primer pairs #5/#9 and #6/#10 with the appropriate clones. Such duplication confirmed the authenticity of the original reaction products. The two amplified fragments were subcloned into M13 vectors for sequence determination.

It appeared that the clones represented two parts of the α-subunit gene, λE6 containing the 57-exon and upstream sequences and λE4 containing the 108-exon and downstream sequences. The two clones, however, failed to cross-hybridize, indicating that they do not overlap and that, therefore, the region of the gene between the 57- and 108-exons was incomplete. A new library was constructed in λGEM11 (see Materials & Methods) and screened as before with the PHα57 probe. A single clone, λ3551, was isolated and again subjected to southern analysis with the 57-and 108-specific oligonucleotides. As shown in Figure 5.4, an 11 kb fragment resulting from digestion of λ3551 with *Sst*I and *Eco*RI hybridized to both probes. The fact that λ3551 carries both the 57 and 108 exons together with their intervening region clearly establishes that these two sequences originate from a common gene as alternatively spliced exons. A gene map resulting from extensive restriction digest and southern blot analysis is presented in Figure 5.5. The size of the region between the 57 and 108 exons was determined to be 7.5 kb which explains why no amplification products of this part of the gene could be obtained.

D. GENERATION OF THE PHα108 MESSAGE INVOLVES ATYPICAL SPLICING

The nucleotide sequences at the exon-intron boundaries (Figure 5.6) were established by sequence comparison of cDNA and

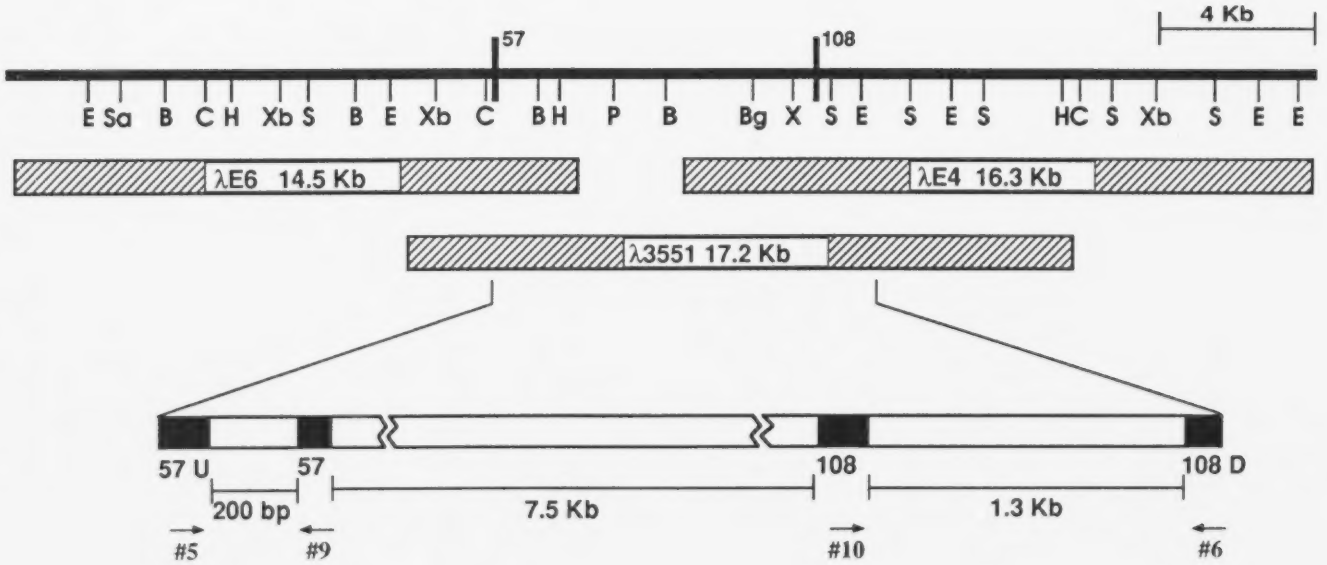
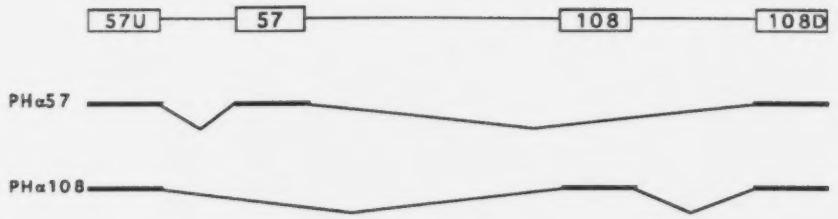


Figure 5.5. Restriction endonuclease mapping of the overlapping clones. The three genomic clones were extensively analyzed by restriction digestion and hybridization to create a composite map of the genomic locus. Placed below the main map are the relative positions of the clones. The sizes of the genomic inserts are indicated. Part of the overlapping clone λ3551 is magnified to show the 57/108 alternatively spliced region. Restriction sites within the gene are; *Eco*RI (E), *Sst*I (S), *Sal*I (Sa), *Bam*HI (B), *Cla*I (C), *Hin*DIII (H), *Xba*I (Xb), *Xho*I (X) and *Bgl*II (Bg). The positions of oligonucleotides used in PCR analyses (#5, #6, #9 and #10) are shown below the enlarged section of the map.

(a)



(b)

Sequence at exon–intron junctions				
mRNA	Exon	5' Splice Donor	3' Splice Acceptor	Exon
PHα57	57U	GTC ATC AAT gtgagt	attacttctcttcag CCT GGA GAA	57
	57	CGA ATT G gtgagt	ttctccttctttaag GT ATC GTG	108D
PHα108	57U	GTC ATC AAT gtgagt	tcttctctctttcag AGA TTT CTT	108
	108	CAT ATG ataagt	tttctccttctttaa GGT ATC GTG	108D
Consensus		Exon—gtaagt	(Py)n (Py)ag—Exon	

Figure 5.6. Alternative splice pathways and exon-intron splice sites for PHα57 and PHα108. *(a)* Exon-intron organization of part of the gene for the α-subunit of succinyl-CoA synthetase. Exons are indicated by open boxes. The two pathways giving rise to PHα57 and PHα108 are depicted below. *(b)* Exon-intron splice sites used to generate the two mRNAs. Exon sequences are in capital letters, while intron sequences are in the lowercase. Consensus splice donors and acceptors are shown at the bottom. Py designates pyrimidine.

genomic clones. With one exception, the 5' donor and 3' acceptor splice sites of the introns conform to the GT—AG rule [31]. One deviation from this rule was found at the 5' donor site downstream of exon 108 where an AT was apparently utilized. Two other examples of 5' donor sites utilizing the nucleotides AT have been found in the genes for human proliferating cell nucleolar protein, p120 [32], and chicken cartilage matrix protein [33]. The remainder of the sequence at this junction agrees well with the consensus sequence for 5' donor sites [34].

One of the 3' acceptor sites is being differentially utilized by the two mutually exclusive exons, 57 and 108. The AG at this site provides the perfect junction for splicing of the 57 exon. In order to maintain reading frame, the 108 exon would be required to use the splice sequence AA rather than the typical AG at this splice acceptor. This further compounds the problem for splicing of the 108 exon. It follows that since AG forms the more preferred splice acceptor, the 57-spliced variants would be expected to predominate. This is consistent with our findings that out of 25 cDNAs examined, only 3 were demonstrated to contain sequences characteristic of the 108 exon. It is conceivable that differential usage of this acceptor site could represent an important control point regulating the splicing of the two mRNA species.

IV. DISCUSSION

Two cDNAs encoding different forms of the α-subunit of pig heart succinyl-CoA synthetase have been described here. The

sequence derived from one of these, PHα108, contains a unique stretch of 108 nucleotides within its open reading frame The 108 nucleotide stretch replaced a shorter 57 nucleotide sequence in PHα57. The 36 amino acids encoded by the 108 sequence bear no resemblance to the corresponding region encoded by PHα57 and are not seen in any other α-subunits [18,29-30]. Thus, the protein encoded by PHα108 represents a novel form of the α-subunit. Overall, these findings are consistent with the various reports of polymorphism in the pig heart enzyme [12-14]. The mRNAs that encode the two forms of the α-subunit are the result of a mutually exclusive splicing event. Such alternative splicing is commonly used in eukaryotes to generate isoform diversity in proteins [36]. The generation of pig heart isoforms by alternative splicing contrasts with an earlier report suggesting the presence of more than one gene for the α-subunit of the yeast enzyme [30].

The generation of the PHα108 message involves atypical donor and acceptor splice sites. The use of AT in place of the preferred GT [31] at positions +1 and +2 of the donor site has only been reported to occur in two other genes [32, 33]. However, five of the first six nucleotides at the 5' end of the intron are identical to the consensus sequence for a donor splice site [34]. It has been noted that where non-consensus 5' splice sites are used, the nucleotide G at position -1 matches the consensus in 96% of cases [35]. The donor site of the 108 exon obeys this rule. Such adherence to the remainder of the consensus may make use of this site possible. Both the 57 and the 108 sequence are spliced to a common 3' acceptor site. This site is being differentially utilized by the two sequences. In order for the

108 sequence to remain in frame with the downstream part of the message it must use the dinucleotide AA rather than the preferred AG (used by the 57 sequence) at the acceptor site. Interestingly, all cases where non-consensus acceptor sites are used involve differential splice site selection [35]. Moreover, use of adenosine in place of guanosine at both sites preserves the purine nature of the nucleotide involved in the splicing reaction.

Preliminary northern blot analysis shows evidence of two transcripts for the α-subunit that were 1.8 kb and 1.6 kb in size. It has not yet been determined whether either of these species contain the unique 108 sequence. The longest sequence obtained in this study was 1.3 kb and contained only 42 nucleotides of 5' non-coding sequence. Since all cDNAs examined show identical 3' ends, the lengths of these transcripts suggest that the 5' untranslated region of our cDNAs is incomplete. This is not surprising given that the corresponding sequence from rat liver contains 500 nucleotides upstream from the ATG [18].

In a separate study, a cDNA encoding the β-subunit was isolated from the same library [16]. This sequence can, now, be expressed in *E. coli* in combination with each of the α-subunit cDNAs. Such expression will allow the purification and biochemical characterization of these two forms of the protein. Moreover, the functional significance of the variant sequence in the α-subunit can be assessed.

Acknowledgments

The contents of this chapter resulted from collaboration with Dr. Tian Wei Lin. Dr. Lin was responsible for the initial discovery of the two cDNAs and carried out the PCR work. Dr. Lin also isolated the genomic clones, λE4 and λE6, from a commercial library. The author characterized the remaining cDNAs, provided the DNA for PCR, created the second genomic library and isolated the overlapping clone, λ3551. All Southern and Northern blots were performed by the author. The gene map was entirely generated from the author's work. Warren D. Henning was responsible for carrying out much of the sequence determination. This author is entirely responsible for the presentation of all the data in this thesis.

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Chapter 6

The two forms of the pig heart α-subunit support GTP-specific enzyme activity

I. INTRODUCTION

In addition to its position within the tricarboxylic acid (TCA) cycle, mammalian succinyl-CoA synthetase plays a role in ketone body catabolism [1] and porphyrin synthesis [2]. In the TCA cycle, the enzyme catalyzes the conversion of succinyl-CoA to succinate with concomitant GTP production. In contrast, ketone body breakdown requires high levels of succinyl-CoA to be maintained [3]. Ottaway and colleagues [1,4] suggest that such opposing functional demands on the enzyme-catalyzed reaction could best be accommodated by two distinct enzymes.

Both ATP- and GTP-specific enzymes have been found in tissues from a wide range of animal species including mammals [4-6]. Moreover, the ratio of these two activities varied depending on the tissue source [5,6]. Weitzman *et al.* [6] showed that these activities resulted from two distinct and separable enzymes. It had been suggested that a GTP-specific enzyme, operating under high GTP/GDP ratios (~100/1), would maintain favorably high levels of succinyl-CoA for ketone body metabolism [1]. In support of this proposal, Jenkins and Weitzman [7] demonstrated that the level of GTP-specific activity was significantly increased in tissues of ketotic animals. In a separate study, two distinct GTP-specific enzymes were seen in mouse liver [2]. The two forms could be distinguished by their pH profiles and by their behavior to cellulose acetate electrophoresis. One of these enzyme forms was elevated threefold under conditions of increased porphyrin synthesis. These reports highlight the role of GTP-specific enzymes in ketone body metabolism and porphyrin

synthesis and suggest that the ATP-specific enzyme might be associated with the TCA cycle. In Trypanosomes, elevated TCA cycle activity is accompanied by increases in ATP-specific activity [8].

The isolation of cDNAs encoding two different forms of the α-subunit of the pig heart enzyme (Chapter 5) is consistent with the reports of polymorphism. Separate studies have identified a cDNA for the β-subunit of the enzyme [9]. Enzymes resulting from combinations of each of the α-subunits with the β-subunit have been made. This report describes the production and characterization of the two forms of the pig heart enzyme.

II. MATERIALS AND METHODS

A. Bacterial strains, enzymes and reagents

The following *E. coli* strains were used in this study: JM109 was routinely used for the construction and propagation of M13 and plasmid derivatives. BL21(DE3) was used for the expression studies. The genotypes of these strains can be found in **Appendix 1**. Kinase, ligase, Klenow DNA polymerase and restriction enzymes were supplied by Bethesda Research Laboratories and New England Biolabs. Reagents for sequencing were purchased from United States Biochemicals. All other chemicals were supplied by Sigma and British Drud House.

B. Construction of expression plasmids

The plasmid, pGS202, carries the genes for the two subunits of the *E. coli* enzyme [10]. The two genes overlap by a single base-pair

such that production of their encoded subunits is coupled [10,11]. The sequence responsible for coupling translation lies between an *Esp*I site at the 3' end of the β-subunit gene and a *Cla*I site at the 5' end of the α-subunit gene. The reader is referred to Chapter 7 for further details.

The *E. coli* genes were placed under the control of a T7 promoter in the vector, pT7-6 [12], generating pT7 202 (Fig. 6.1). The *E. coli* genes were sequentially replaced by their corresponding pig heart sequences while preserving the region responsible for translational coupling (Fig. 6.2 and 6.3). The two pig heart cDNAs, PHα57 and PHα108, were modified by creating a *Cla*I* site at a position equivalent to that of the *E. coli* gene. The modified sequences were cloned into pT7 202, in place of the corresponding *E. coli* gene, creating pT7 202/α57 and pT7 202/α108 (Fig. 6.2). Since *E. coli* subunits do not functionally interact with mammalian subunits (Brownie *et al.*, unpublished results), these vectors were used as negative controls in expression experiments. A cDNA encoding the pig heart β-subunit [9] was modified both at the 5' and 3' end of its open reading frame. An *Nco*I* site was incorporated into the initiator codon of the sequence allowing the 5' end of the cDNA to be fused to the *atp*E RBS in pT7-6 RBS (Fig. 6.3). An *Esp*I* site was created in the pig heart sequence at a position equivalent to that of the *E. coli* gene. The *E. coli* β-subunit gene in pT7 202/α57 and pT7 202/α108 was replaced by the corresponding pig heart sequence, generating pT7 β/α57 and pT7 β/α108, respectively (Fig. 6.3).

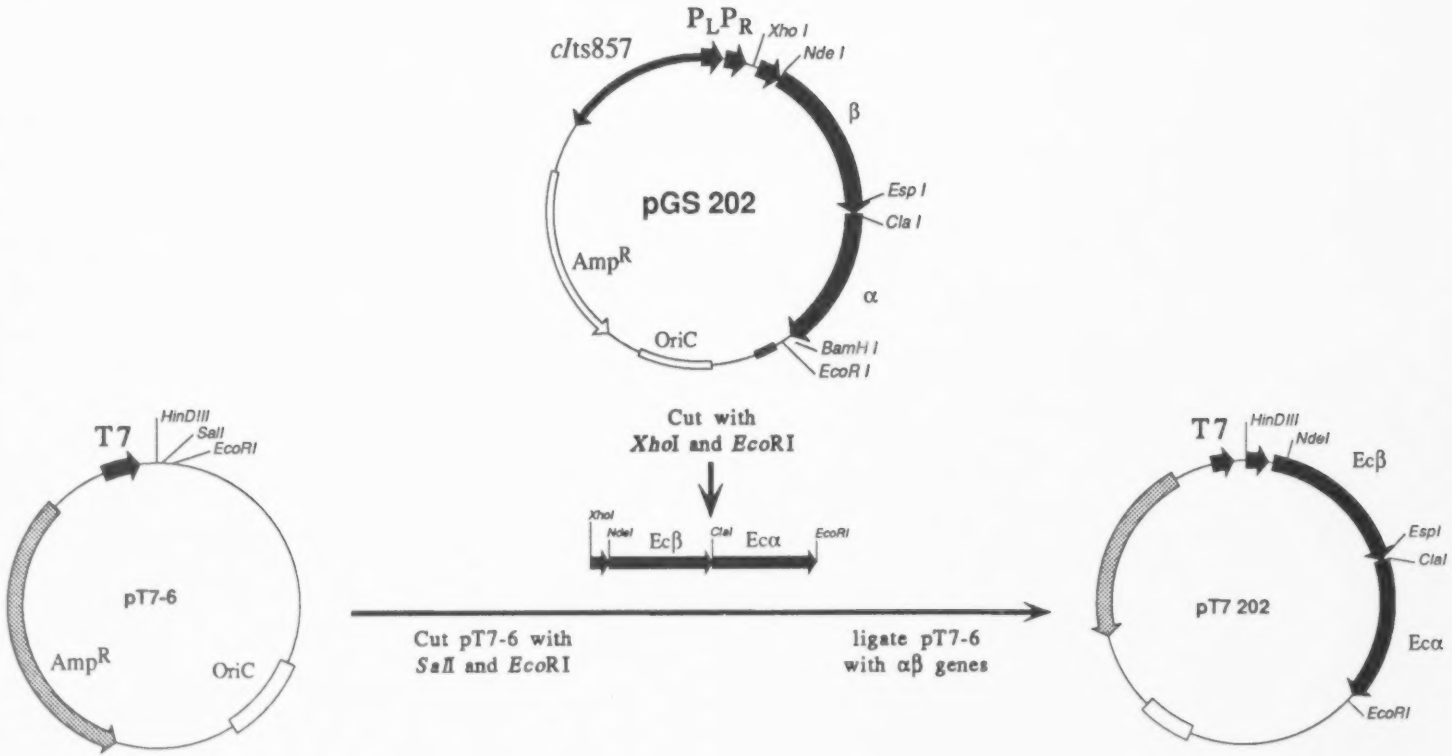


Figure 6.1. Construction of an expression plasmid for production of pig heart succinyl-CoA synthetase. I. pGS202 carries the genes for the α- and β-subunits of *E. coli* succinyl-CoA synthetase. In this plasmid, the genes are under the control of a $\lambda P_L P_R$ promoter and the translation initiator from the *atp*E gene (three short black arrows). The genes were transferred, together with the translation control region, to the plasmid, pT7-6, placing them under the control of a strong T7ϕ*10* promoter.

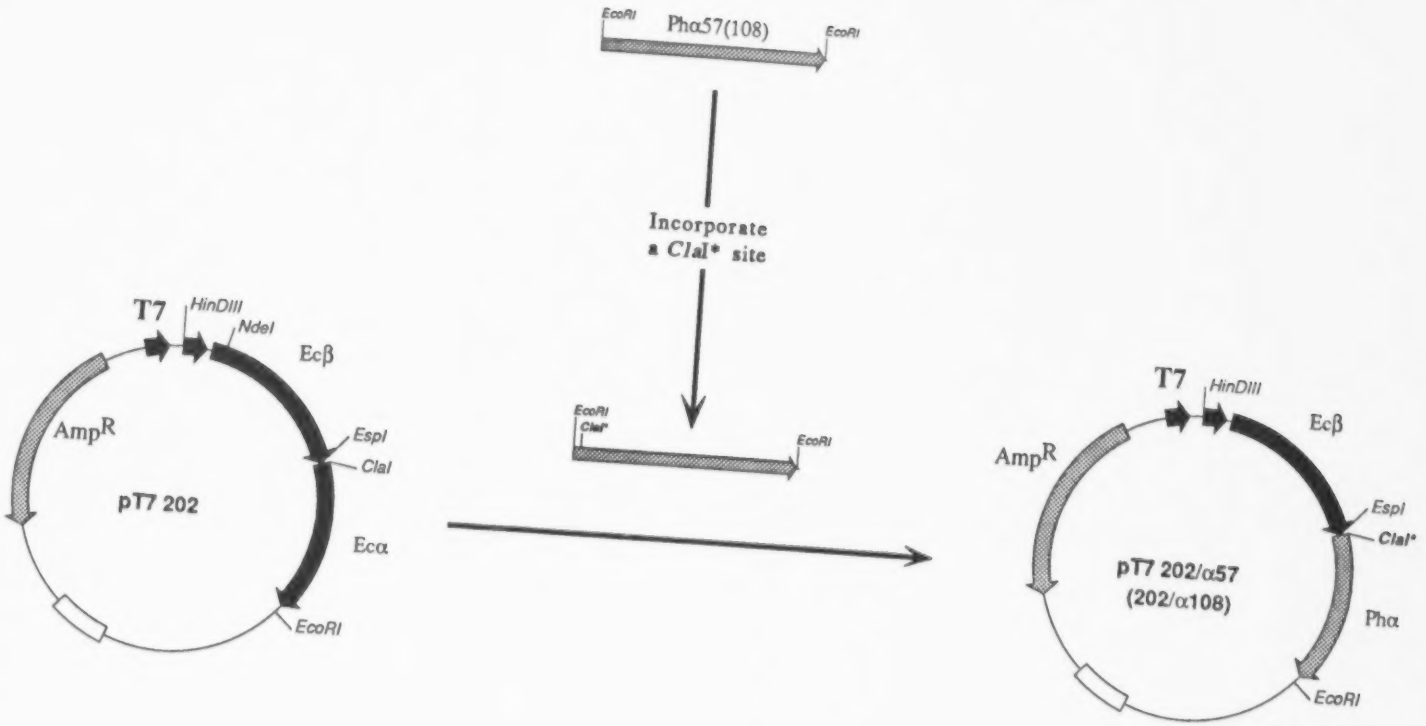


Figure 6.2. Construction of an expression plasmid for production of pig heart succinyl-CoA synthetase. II. The gene encoding the α-subunit of the *E. coli* enzyme in pT7 202 was replaced with the corresponding sequences for the pig heart subunit (Phα57 and Phα108) creating the hybrid plasmids, pT7 202/α57 and pT7 202/α108. The 5' end of the open reading frames in Phα57 and Phα108 were altered to include a ***Cla*I** restriction site marked by an asterix (*). This allowed for the in-frame fusion of the pig heart sequences to the 5' end of the *E. coli* α-subunit gene. The sequence of the oligonucleotide used to create this site is given in **Appendix II, #11.**

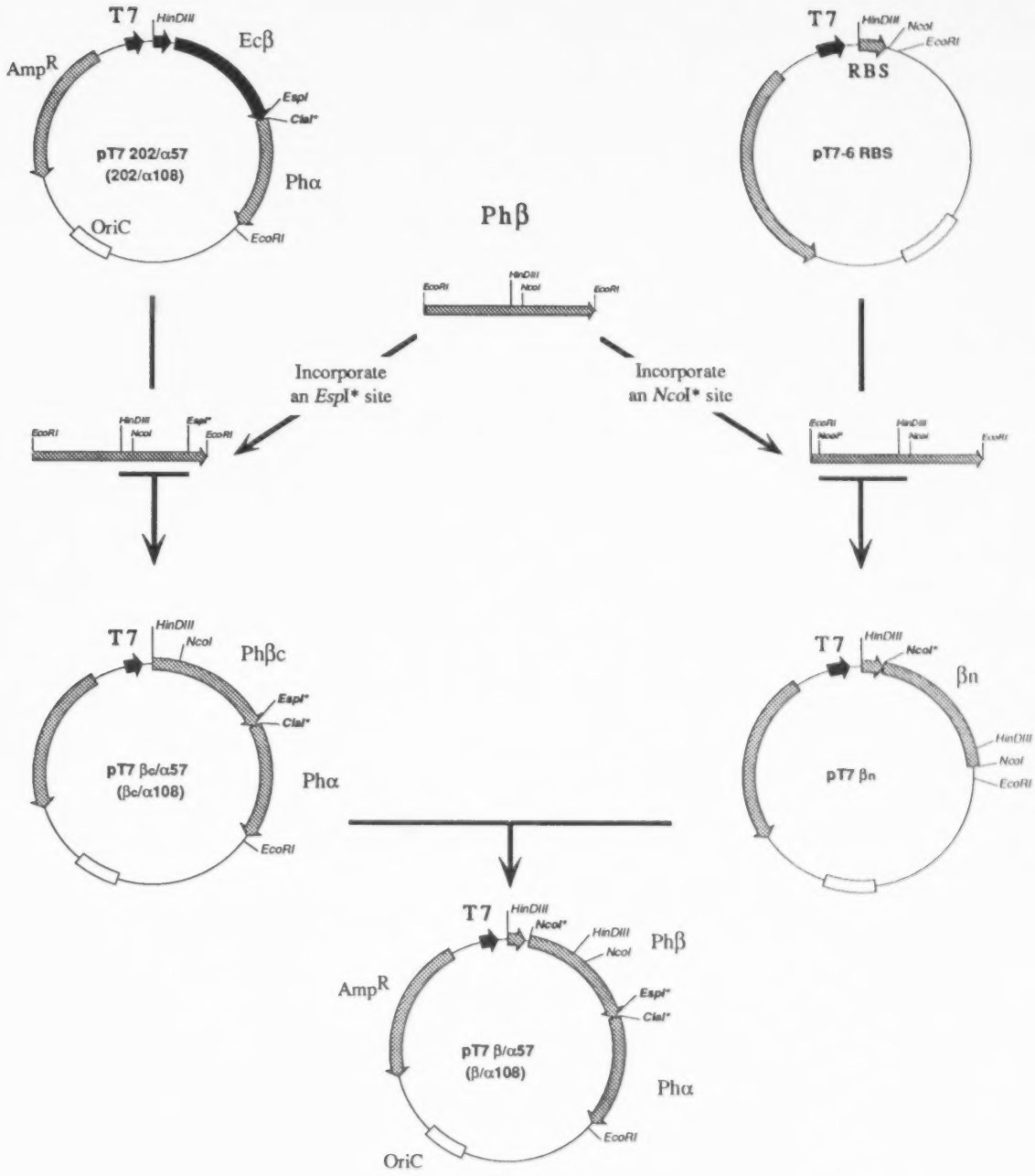


Figure 6.3. Construction of an expression plasmid for production of pig heart succinyl-CoA synthetase. III. The gene encoding the β-subunit of the *E. coli* enzyme in pT7 202/α57 and pT7 202/α108 was replaced with the corresponding sequence for the pig heart subunit (**Phβ**), creating pT7 β/α57 and pT7 β/α108. Both the 5' and 3' end of the open reading frame in Phβ were altered to include the restriction sites, ***Esp*I** and ***Nco*I**, marked by an asterix (*). This allowed for the in-frame fusion of the pig heart sequence to both the 3' end of the *E. coli* β-subunit gene and to a ribosome binding sequence (**RBS**) in pT7-6RBS. The sequences of the oligonucleotides used to create these sites are given in **Appendix II, #12,#13.**

All genetic manipulations were performed according to standard protocols [13]. Sequence alterations were made using site-directed mutagenesis methods of Zoller and Smith [14] on uracil-enriched templates [15]. Mutations were made in M13mp18 derivatives, identified by restriction digest analysis and confirmed by dideoxynucleotide sequencing [16]. Sequences of the oligonucleotides used to create *Cla*I*, *Nco*I* and *Esp*I* restriction sites are given in **Appendix II** as #11, #12 and #13, respectively.

C. Expression

Cultures of BL21(DE3) carrying expression plasmids were grown up from freshly transformed cells at 37 °C in LB broth supplemented with ampicillin (50 µg/ml). See **Appendix III** for medium composition. When the A_{600} of the culture reached ~0.6, expression was induced by the addition of IPTG to a final concentration of 0.5 mM. In the case of large scale cultures a final concentration of 0.1 mM was found to be adequate. The cultures were incubated for 5 hours at 37 °C following induction. Cells were harvested by centrifugation and lysed by sonication. Succinyl-CoA synthetase activity was measured by the spectrophotometric method of Cha [17].

D. Enzyme fractionation

Cell pellets obtained from 2.5 liter cultures were resuspended in 100 ml of sonication buffer (50 mM potassium phosphate, pH 7.4, 1 mM EDTA, 0.1 mM PMSF). The suspension was sonicated and the resulting lysate was centrifuged at 15,000xg for 30 minutes, 0 °C. Proteins, precipitated by 15 % ammonium sulfate, were

removed by centrifugation at 15,000xg. The supernatant fraction was brought to 40 % ammonium sulfate. Precipitated proteins were collected by centrifugation and solubilized in 10 mM potassium phosphate, pH 7.4, 1 mM EDTA, 0.1 mM PMSF. The sample was passed through a 1.25 x 30 cm Sephadex G25 column using the solubilizing buffer as the elution buffer. Fractions containing high enzyme activity were pooled and directly loaded on a 50 ml hydroxyapatite column equilibrated with the same buffer. The column was washed with a further 100 ml of equilibration buffer. Two columns were set up in this manner, one for purification of $\beta/\alpha 57$ and one for $\beta/\alpha 108$. Proteins were eluted from the two columns simultaneously using a common buffer gradient. Elution was carried out with a 500 ml gradient of 10 mM to 400 mM potassium phosphate, pH 7.4. For Affigel Blue chromatography, two 1.25 x 20 cm columns were equilibrated with 50 mM potassium phosphate, 1 mM EDTA, 0.1 mM PMSF, pH 7.4. Pooled fractions eluted from the Sephadex G25 column were passed through the Affigel Blue column and washed with 100 ml of equilibration buffer. Elution was with a linear salt gradient from 0 to 2 M KCl in 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 0.1 mM PMSF. Proteins that had been subjected to ammonium sulfate fractionation were passed through Sephacryl S200 gel filtration medium using the same buffers as described for gel filtration through Sephadex G25. Chromatographic matrices were purchased from Pharmacia and Biorad. The preparation and use of these reagents are described in **Appendix IV**.

III. RESULTS

A. Production of two forms of the pig heart enzyme

The molecular masses of the two pig heart α-subunits, α57 and α108, were calculated to be 32.11 kDa and 34.45 kDa, respectively. The size of α57 corresponds to that estimated for the α-subunit from purified pig heart enzyme [18]. The α57 protein produced in *E. coli* migrated alongside the α-subunit of a purified enzyme on SDS-PAGE, while α108 protein ran significantly more slowly. In addition, protein sequence obtained from the α-subunit of the purified enzyme showed it to be similar to the α57 form. The variant sequence present in the α108 subunit is unique and is not found in any other α-subunit whose sequence has been reported [11,19-21].

Expression plasmids were created to direct the production of each of the α-subunits in combination with the pig heart β-subunit. A plasmid construct, pT7 202, capable of producing high levels of the *E. coli* enzyme, was chosen as a framework on which to build a similar expression system for the pig heart enzymes. The two *E. coli* genes overlap by one base-pair such that production of their encoded subunits is coupled. When BL21(DE3) cells containing this plasmid are induced, both α- and β-subunits are seen to accumulate at similar levels over time (Fig. 6.4*a*). The gene for the α-subunit in pT7 202 was replaced by either PHα57 or PHα108 sequences creating the plasmids, pT7 202/α57 and pT7 202/α108, respectively. BL21(DE3) cells containing these plasmids were found to produce high levels of both pig heart α-subunits (Fig. 6.4*b*). Furthermore, the levels of accumulated α-subunit were similar to those of the *E. coli* β-subunit,

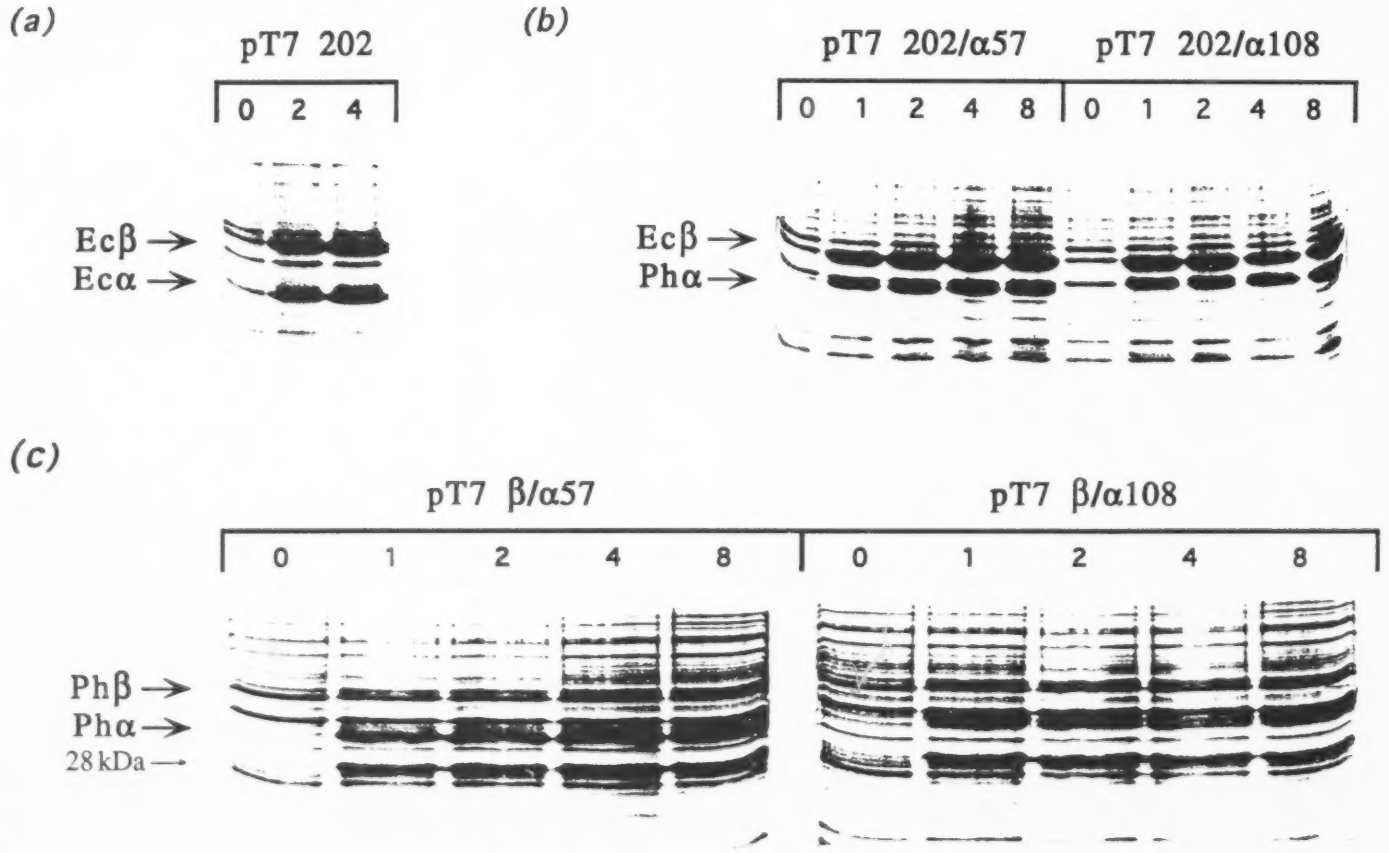


Figure 6.4. Production of subunits of pig heart succinyl-CoA synthetase in *E. coli*. BL21(DE3) cells containing the expression plasmids, pT7 202 in ***(a)***, pT7 202/α57 and pT7 202/α108 in ***(b)*** and pT7 β/α57 and pT7 β/α108 in ***(c)***, were grown in LB broth to an A_{600} of 0.5. Expression was induced by the addition of IPTG. See **Materials and Methods** for details. Samples were withdrawn from the cultures at 0, 1, 2, 4 and 8 hours following the induction, centrifuged and the cell pellets were lysed in SDS-PAGE running buffer. Samples were analyzed by SDS-PAGE followed by Coomassie staining.

in both cases. This implies that the mechanisms for translational coupling of the two subunits are still operating in these plasmids.

The sequence for the pig heart β-subunit replaced the gene for the *E. coli* β-subunit in pT7 β/α57 and pT7 β/α108. These plasmid constructs were expected to produce both subunits of the pig heart enzyme in BL21(DE3). As with the parent plasmids, high levels of both of the α-subunits accumulated following induction of cultures (Fig. 6.4*c*). However, very low levels of the pig heart β-subunit were seen in both cultures. A 28 kDa protein was found to accumulate at levels similar to the α-subunits, in place of the expected β-subunit (see Chapter 7 for further details).

B. The two forms of the enzyme are GTP-specific

BL21(DE3) cultures containing the pT7 β/α57 and pT7 β/α108 plasmids were examined for succinyl-CoA synthetase activity. Despite the poor levels of the β-subunit, both cultures showed elevated levels of GTP-specific enzyme activity following induction (Fig. 6.5*a* and *b*). The host enzyme can use either ATP or GTP as a substrate [22]. This is reflected in the activities measured for controls, cultures of BL21(DE3) cells containing either pT7 202/α57 or pT7 202/α108. The ATP- and GTP-specific activities measured at the time of induction and 5 hours after induction remained almost constant. The ratios of GTP- to ATP-specific activity were about 1:3 before or after induction, consistent with activity of the host enzyme. In contrast, the GTP-specific activity produced by BL21(DE3) cells containing the pT7 β/α57 and pT7 β/α108 plasmids increased by a factor of two over the course of the induction period. The ATP-

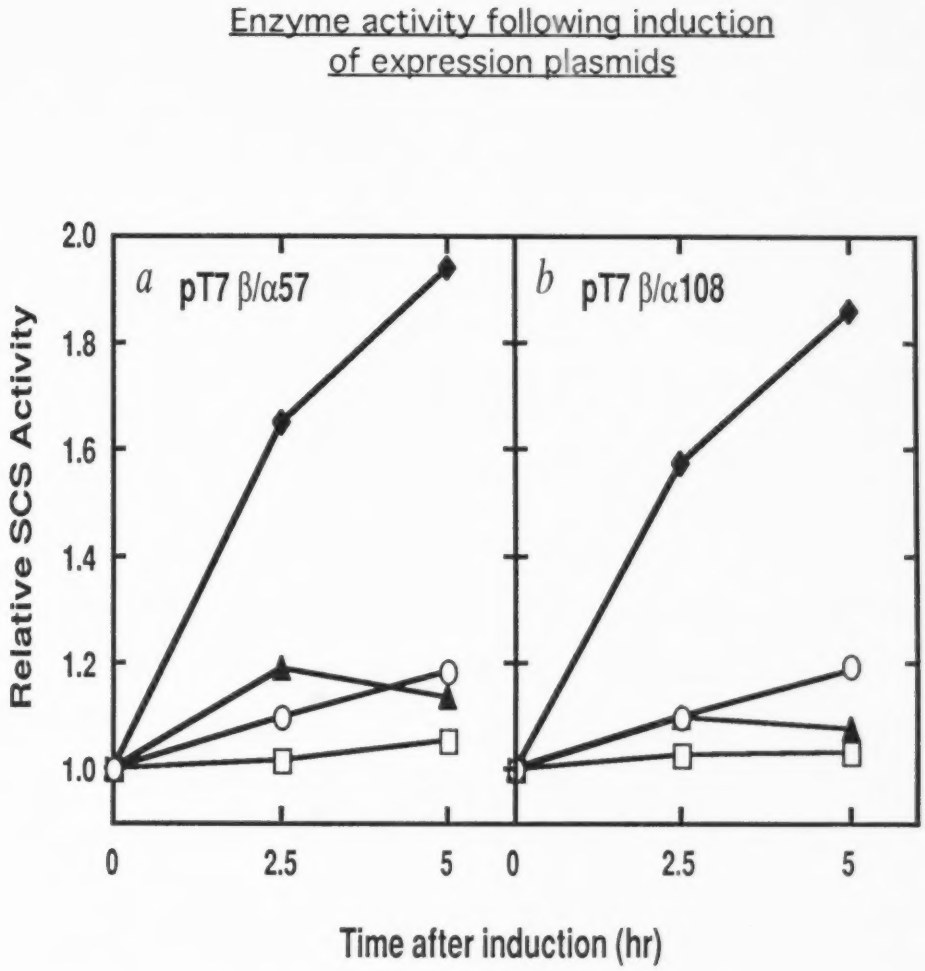


Figure 6.5. Succinyl-CoA synthetase activity in lysates of *E. coli* expressing pT7 β/α57 and pT7 β/α108. α57 and α108 were produced with either the *E.coli* β-subunit in pT7 202/α57(α108) or the pig heart β-subunit in pT7 β/α57(α108). Samples were removed at the time of induction and at timed intervals afterward. Lysed cells were assayed for succinyl-CoA synthetase activity using either ATP or GTP as substrate, and are shown as specific activities relative to those measured at the time of induction due to host enzyme. Open symbols: ATP as substrate; closed symbols: GTP as substrate. ***(a)*** (□,▲): pT7 202/α57 (control); (O,◆): pT7 β/α57 ***(b)*** (□,▲): pT7 202/α108 (control); (O,◆): pT7 β/α108.

specific activity in these cultures, like the controls, stayed essentially constant. Thus, it is concluded that these cultures are producing the two forms of the pig heart enzyme, both of which exhibit GTP specificity.

C. Separation of the two forms on hydroxyapatite

Cell extracts from 2.5 liter cultures producing the two forms of the pig heart enzyme, either β/α57 or β/α108, were fractionated on hydroxyapatite columns. This chromatography matrix proves to be an excellent medium for separation of the mammalian and bacterial enzymes. The bacterial enzyme typically elutes with 65 mM phosphate, while the mammalian enzyme, as purified from pig heart, emerges at 200 mM phosphate. Extracts containing β/α57 and β/α108 were loaded onto two 1.25 x 30 cm columns of hydroxyapatite. Proteins were eluted from these columns simultaneously using a common gradient of potassium phosphate. See **Materials and Methods** for more details. The profiles of succinyl-CoA synthetase activity, both ATP- and GTP-specific, from these two fractionations are presented in Figure 6.6*a* and *b*.

In both cases two separate peaks of enzyme activity were clearly identified. The first peak of enzyme activity eluted from both columns exhibited specificity for both ATP and GTP, a characteristic of the host *E. coli* enzyme. Moreover, the position of this peak is consistent with it being of bacterial origin. The second peak of activity in both cases showed an absolute specificity for GTP. The enzyme specific to the β/α57 combination began to emerge from the column at 200 mM phosphate (Fig. 6.6*a*). This corresponds with the

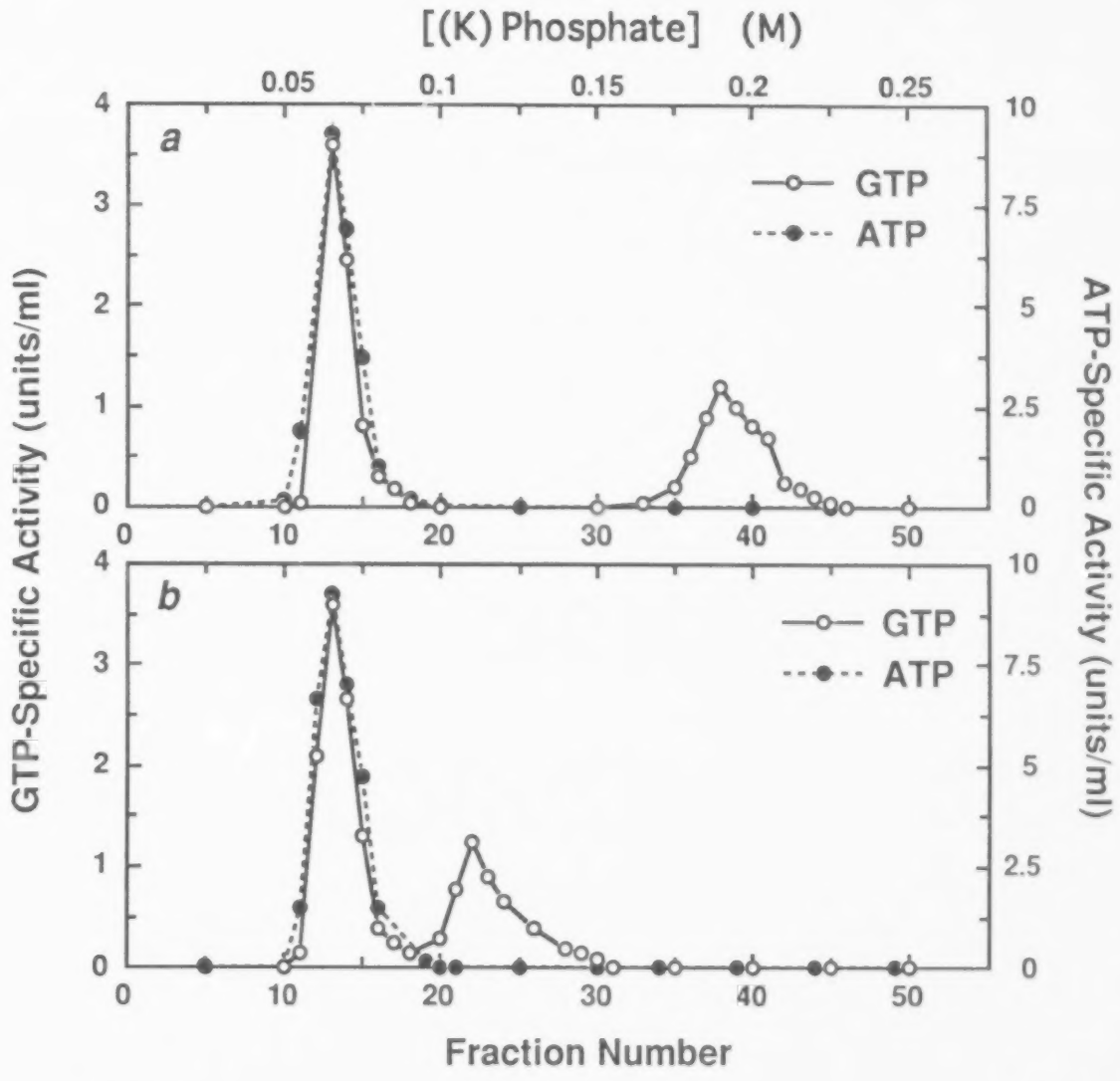


Figure 6.6. Elution profiles of the two isoforms from hydroxyapatite columns. Extracts from bacterial cultures synthesizing each of the α-subunits with a pig heart β-subunit were fractionated on hydroxyapatite as stated in Materials and Methods. A 10-400 mM K phosphate gradient was used, and 80 x 2 ml fractions were collected from each column. ATP- and GTP-specific enzyme activities were determined in both cases. Activity profiles for the first 50 fractions of both isoforms, β/α57 in ***(a)*** and β/α108 in ***(b)*** are shown here.

position at which purified pig heart enzyme elutes, confirming the similarity between the β/α57 form and the purified enzyme. In contrast, the GTP-specific enzyme from the β/α108 combination (Fig. 6.6*b*) emerged at an intermediate (100 mM) phosphate concentration. Thus, hydroxyapatite is capable of distinguishing between the two forms of the pig heart enzyme, a property that would be useful for separating the equivalent forms from pig heart tissue.

D. Purification of the enzyme from pig hearts

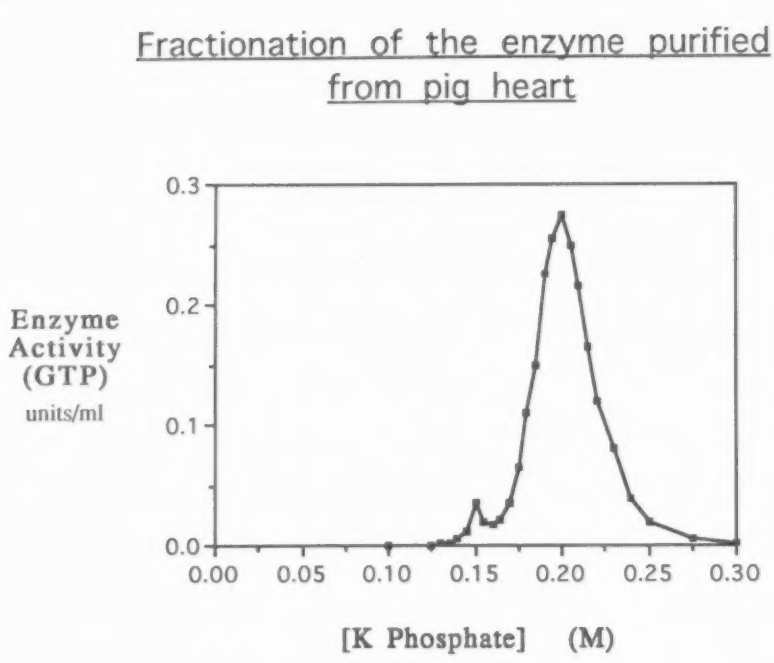
The behavior of the β/α108 enzyme on hydroxyapatite is similar to a GTP-specific activity previously identified by Cha *et al.* [23]. In the first report describing the use of a hydroxyapatite column for the purification of pig heart succinyl-CoA synthetase, they noticed a minor peak of enzyme activity emerging early in the phosphate gradient at a point similar to where the β/α108 is found to elute. This was followed by the main peak of activity corresponding to the β/α57 form of the enzyme. Thus, the hydroxyapatite matrix can identify different forms of the pig heart enzyme.

The enzyme was purified from a sample of heart tissue taken from one week old piglets. One week old piglets were the original source of the RNA used to create the cDNA library from which PHα108 was isolated. When β/α57 and β/α108 forms were subjected to gel filtration on Sephacryl S200, both migrated at rates expected of dimers with a molecular mass of ~70 kDa. In addition, both forms behaved similarly to purification on Affigel Blue. Since both forms of the enzyme copurify on these columns, the extract from the pig heart

tissue was first subjected to fractionation on these matrices to obtain a "semi-pure" preparation that would contain these two forms. Pig heart tissue (5g) was homogenized and filtered through several layers of cheesecloth. The soluble proteins were fractionated with ammonium sulphate as outlined in **Materials and Methods**. The sample was subjected purification by Affigel Blue and Sephadex S200 chromatography followed by fractionation on hydroxyapatite (**Materials and Methods**). Proteins were eluted from the column as before with a 10 mM to 400 mM phosphate gradient. The profile of enzyme activity resulting from this fractionation is shown in Figure 6.7*a*. Two peaks of GTP-specific activity can be seen in this profile. The first peak of activity begins to emerge from the column at ~140 mM phosphate. This peak represents a shoulder on the major peak that follows. The major enzyme form elutes at 200 mM phosphate as expected for the pig heart enzyme. The elution position of the minor enzyme form corresponds to that of the β/α108. This minor peak has been noted in other enzyme preparations and usually represents about 5 % of the total activity.

A peptide, ThrGlnGlnGluGluTyrArgLys, representing part of the variant sequence present in α108 was synthesized and used to raise polyclonal antibodies that would be specific for the α108-related protein. Fractions containing both peaks of enzyme activity were subjected to western blot analysis with these antibodies (Fig. 6.7*b*). Immunoreactive proteins were found in those fractions representing the first peak of enzyme activity and were not seen with the fractions representing the main peak of enzyme activity. Furthermore, the levels of this immunoreactive protein reached a

(a)



(b)

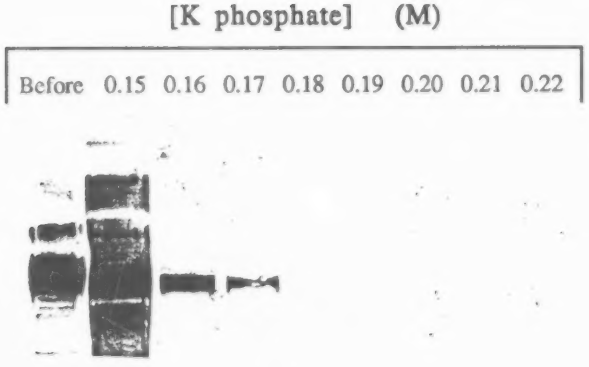


Figure 6.7. Demonstration of a 108-related isoform in purified preparations of pig heart succinyl-CoA synthetase. Succinyl-CoA synthetase was purified from pig heart tissue by ammonium sulfate fractionation and chromatography through Sephacryl S200 and Affigel Blue resins. See **Appendix IV** for details on the use of these resins. This preparation was subjected to fractionation with hydroxyapatite. Proteins were eluted from the resin by application of a 10-400 mM K phosphate gradient. A profile of the GTP-specific activity present in the eluted fractions is shown in ***(a)***. Fractions representing the first peak of enzyme activity to elute from the column were resolved by SDS-PAGE, transferred to nitrocellulose and immuno-reacted with antibodies directed against the 108-specific protein sequence ***(b)***. A sample of the enzyme preparation used in the fractionation was included (**Before**).

maximum in the fractions that showed the highest activity. The estimated mass of this protein was ~40 kDa. This is somewhat larger than the size predicted for α108. However, it is possible that the α108 form of the protein could be post-translationally modified in pig heart tissue.

IV. DISCUSSION

Size heterogeneity has often been noticed in the α-subunit from preparations of pig heart succinyl-CoA synthetase (Brownie *et al.*, unpublished results). It was also known that this heterogeneity can be avoided by the use of hydroxyapatite chromatography. It is possible that these preparations contained two distinct forms of the pig heart enzyme prior to hydroxyapatite fractionation, one of which might be related to the β/α108 form produced in this study. Hydroxyapatite chromatography will be useful in further studies of the two forms of the enzyme. The α108 subunit was found to be less stable than the α57 subunit in cell lysates (Ryan *et al.*, unpublished results), a factor that may make its purification from pig heart tissue difficult. However with the information gained in these studies it should be possible to specifically purify the α108-related form from mammalian tissue.

Two distinct GTP-specific enzymes have been found in mouse liver [2]. The level of one of these enzymes rose threefold during increased porphyrin synthesis. Interestingly, the two forms could be separated by electrophoresis on cellulose acetate paper. These findings may be relevant to the two GTP-specific forms reported

here. It must be pointed out that the location of the nucleotide binding site is not known. It is, therefore, possible that the GTP specificity of both β/α57 and β/α108 could result from the common β-subunit they possess.

The amino acid sequence of that part of α57 subunit that is different in the α108 subunit is well conserved in all known forms of the enzyme [11,19-21]. In the crystal structure of the *E. coli* protein, this sequence forms an interconnecting structure between the two domains of the α-subunit: the N-domain which binds CoA and the C-domain containing a phosphohistidine residue [24]. ATP-citrate lyase catalyzes a reaction similar to that of succinyl-CoA synthetase. This enzyme shows substantial sequence similarity to the α-subunit [25]. The region in ATP-citrate lyase corresponding to the α57-related sequence shows only partial homology to the α-subunit. Furthermore, its size is larger due to the insertion of an additional eight amino acid residues. Thus, there is precedence for both sequence and size variation in this region of a related protein.

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Chapter 7

Optimizing production of the pig heart enzyme in *E. coli*.

I. INTRODUCTION

In *E. coli*, most genes are arranged in clusters and are transcribed in the form of polycistronic messages. These polycistronic mRNAs bear multiple open-reading frames whose respective translational efficiencies are often inter-dependent [1-5]. It is believed that in many cases, terminating ribosomes re-initiate at a start codon very close to the stop codon of the upstream cistron [6]. Furthermore, not only is the tightness of coupling variable, but the stoichiometry of the relative translation rates may be such that more copies of the downstream product are made per unit time than of the upstream product. Varying degrees of translational coupling are evident between the genes of the *atp*, *gal* and *thr* operons [7-9].

Such translationally coupled genes need not overlap although in many cases they do. It is not uncommon to find a one base-pair overlap between the terminator codon of one cistron and the initiator codon of the next (e.g., UGAUG) [6,10]. The genes for *E. coli* succinyl-CoA synthetase overlap in this way [11]. In addition, a ribosome binding site is found several base pairs upstream of the overlap point (Fig. 7.1). The two genes are cotranscribed with the subunits being translated from a single polycistronic message [12]. Thus, it is thought that the gene arrangement serves to coordinate the production of the subunits in equimolar amounts for assembly. Similar overlapping is seen with the succinyl-CoA synthetase genes from other bacterial species [13,14].

Using the bacterial gene arrangement as a framework, plasmids were constructed that contained the equivalent sequences for the pig

heart subunits (Chapter 6). Expression of these mammalian sequences resulted in very low levels of enzyme activity (3.0×10^{-3} units/ml culture) being produced, whereas the equivalent expression plasmid for the *E. coli* enzyme produced high levels of activity (750×10^{-3} units/ml culture). While the pig heart α-subunit was efficiently produced, the β-subunit was found in very low levels and an additional 28 kDa protein was found. The following study describes improvements made in the expression of the mammalian sequences.

II. MATERIALS AND METHODS

A. Bacterial strains, plasmids and reagents

The following *E. coli* strains were used in this study: JM109 was employed for all the molecular cloning work and expression was carried out in BL21(DE3). The genotypes of these strains can be found in **Appendix I**. The plasmid, pGS202, was provided by Dr. John Guest, University of Leeds, UK [15]. pT7-6 was a gift from Dr. Stan Tabor, Harvard University, USA [16]. Restriction endonucleases, ligase, kinase and polymerases were purchased from Bethesda Research Laboratories and New England Biolabs. Other chemicals and reagents were purchased from Sigma or British Drug House.

B. Vector construction

The plasmid, pGS202, carries the genes for the α- and β-subunit of the *E. coli* enzyme [15]. The genes and upstream *atp*E RBS were removed from this plasmid and placed under the control of a T7

promoter in pT7 202 (Fig. 7.1). The *E. coli* genes were sequentially replaced by sequences encoding the pig heart subunits [Chapter 5,17]. Construction of these plasmids is described in more detail in Chapter 6.

Two sequence alterations were made in the *E. coli* genes. A terminator codon was incorporated into the reading frame of the β-subunit gene using oligonucleotide #14. Sequences of the oligonucleotides are given in **Appendix II**. The resulting sequence produces a 30 kDa N-terminal fragment of the β-subunit. The 3' end of the β-subunit gene and its coupled α-subunit gene were removed and cloned downstream of the atpE RBS in pT7-6. Sequence at the overlap point between the two genes was modified using oligonucleotide #15. The single base-pair overlap was removed and the terminator codon was altered such that the resulting sequence encoded a fusion of the two subunits. The RBS and initiator codon for the α-subunit gene were left in tact. Modifications were made in the expression plasmid containing the pig heart sequences. Sequences involved in internal initiation of translation within the β-subunit gene were altered with oligonucleotide #16. The primary translation initiation site was rebuilt with oligonucleotide #17.

All genetic manipulations were performed according to standard protocols [18]. Sequence alterations were made using the site-directed mutagenesis protocol of Zoller and Smith [19] with modifications by Kunkel *et al.* [20]. Mutations were made in M13mp18 derivatives, identified by restriction endonuclease analysis and confirmed by dideoxynucleotide sequencing [21].

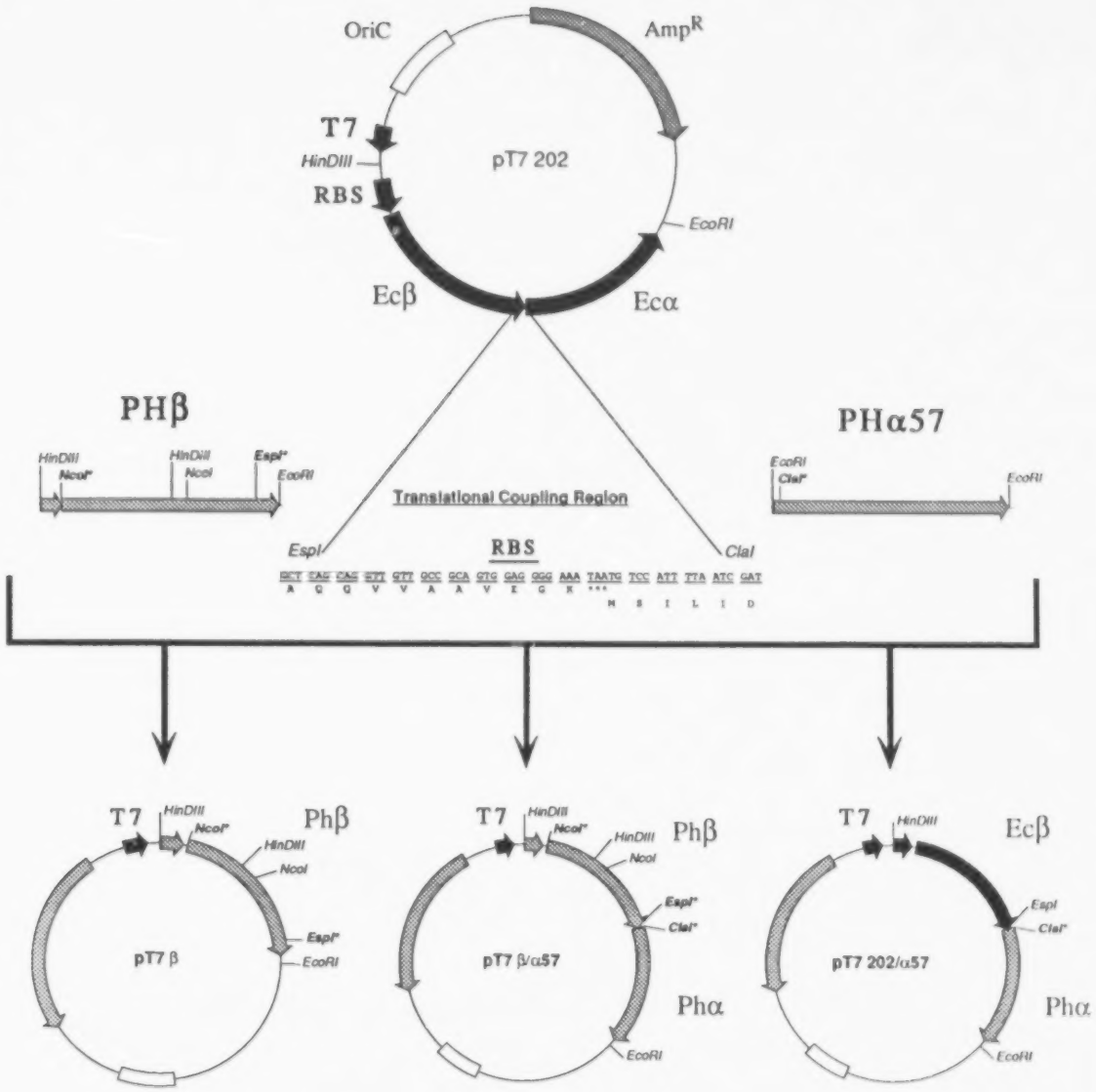


Figure 7.1. Construction of expression plasmids for the production of subunits of mammalian succinyl-CoA synthetase. pT7 202 carries the genes for the *E. coli* α- and β-subunits under the control of a T7 promoter and the ribosome binding sequence (**RBS**) from the *atp*E gene. The open reading frames of the two genes overlap by a single nucleotide to form a region referred to as the Translational Coupling Region. The nucleotide and translated amino acid sequences at the overlap, between the restriction sites *Esp*I and *Cla*I, are shown. The two genes were replaced with sequences encoding the pig heart subunits, PHβ and PHα57, while maintaining the region of the *E. coli* genes at the overlap. New restriction sites were generated in the mammalian sequences to allow for these in-frame fusions. These are shown in *bold type* followed by an asterix (*).

C. EXPRESSION AND WESTERN BLOTTING

Cultures of BL21(DE3) containing pT7 recombinants were grown in LB broth supplemented with ampicillin (50 μg/ml) at 37 °C. Expression was induced by the addition of IPTG to a final concentration of 0.5 mM when the A_{600} reached ~0.6. Cells were harvested after 5 hours and cell lysates were subjected to SDS-PAGE analysis. Protein was transferred to nitrocellulose membranes using a Biorad transfer apparatus. The membranes were incubated with polyclonal antibodies directed against the pig heart enzyme. Blots were developed with secondary antibodies conjugated to alkaline phosphatase. Alternatively, proteins were transferred to polyvinylidene difluoride (PVDF) membranes supplied by Millipore. The proteins were visualized by staining the membrane with 0.1 % Coomassie Blue R in 50 % methanol. The relevant protein was cut from the membrane and subjected to N-terminal sequencing. Succinyl-CoA synthetase activity was assayed using the spectrophotometric method [22].

III. RESULTS

A. TRANSLATIONAL COUPLING IN THE BACTERIAL GENES

BL21(DE3) cells containing pT7 202 were found to express high levels of both the α- and β-subunits of the bacterial enzyme (Fig. 7.2*a*). The levels of accumulation of both subunits increased in a parallel fashion following induction of cells carrying the plasmid. This equivalence in accumulation rates of the subunits was interpreted as evidence that expression of the two genes is coupled.

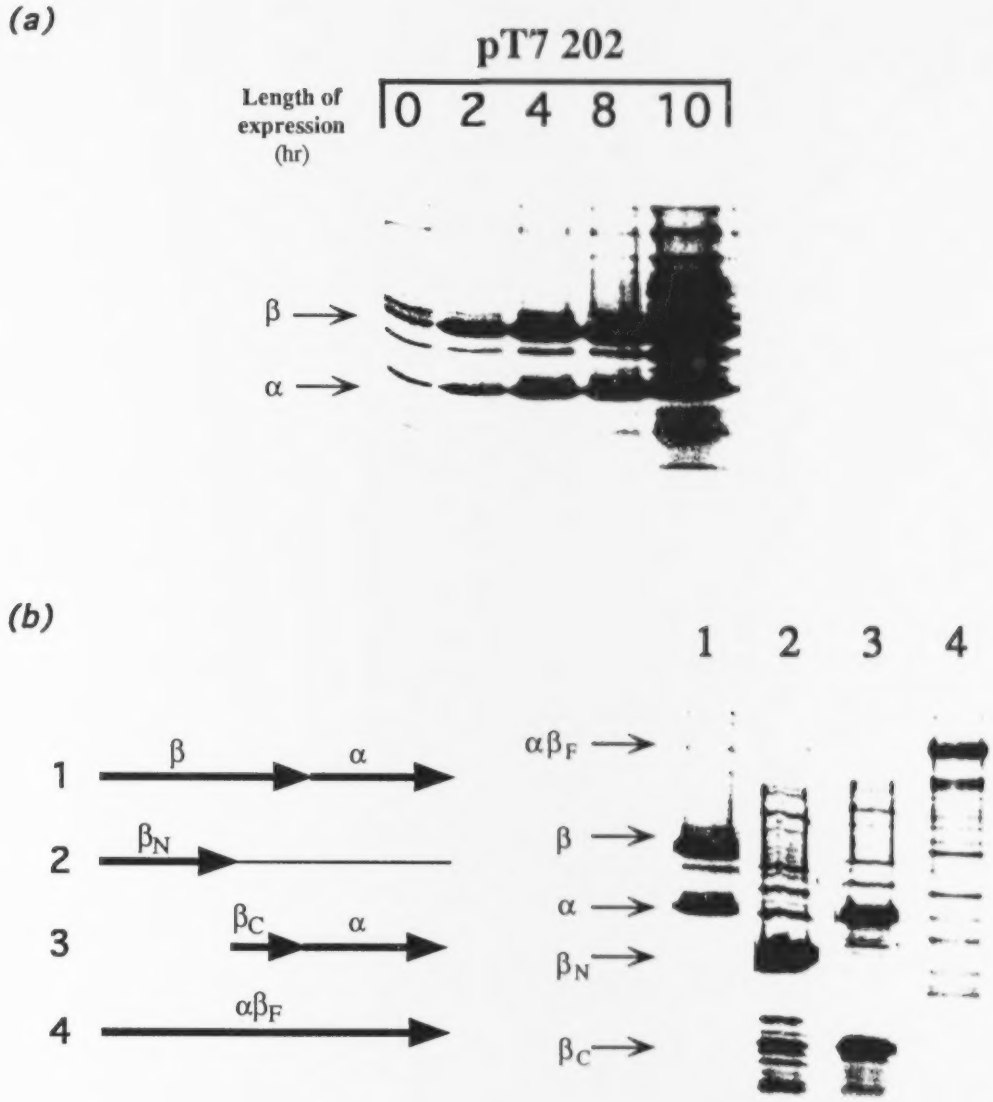


Figure 7.2. Translation of the genes for *E. coli* succinyl-CoA synthetase. ***(a)*** BL21(DE3) cells containing the expression plasmid, pT7 202, were grown in culture to an A_{600} of 0.5 and induced with IPTG. One ml samples were withdrawn at 0, 2, 4, 8 and 10 hours following induction. The samples were centrifuged and cell lysates were resolved by SDS-PAGE. ***(b)*** Derivatives of pT7 202 were created to terminate translation of the β-subunit gene prematurely (2), translate that portion of the β-subunit gene not translated in 2 with the α-subunit gene (3) and produce a fusion of the two subunits (4). Cultures were induced for 5 hrs and cell lysates were analyzed by SDS-PAGE.

Translation of the downstream α-subunit sequence is initiated from a ribosome binding site within the 3' end of the β-subunit reading frame (Fig. 7.1). The gene for the β-subunit was modified to include an in-frame terminator codon, 350 bp upstream from the start for the α-subunit gene. Cells containing this modified form of pT7 202 (β_N) produced high levels of the smaller β-subunit protein but showed no accumulation of the α-subunit (Fig. 7.2*b*). Furthermore, the sequence downstream from this new terminator codon was able to couple the translation of the α-subunit when expressed in a separate pT7 vector ($\beta_C\alpha$). Thus, translation of the α-subunit depends on translation of the 3' end of the β-subunit reading frame. A fusion of the reading frames for the two subunits was made in such a way that the initiator for the α-subunit was not altered. Cells containing this fused sequence in pT7 202 produced high levels of the fusion protein but failed to show any α-subunit protein (Fig. 7.2*b*). Therefore, translation of the β-subunit gene must terminate at its authentic terminator codon for translational of the α-subunit to occur. Taken together, these results demonstrate the coupling nature of translation for coordinating production of the two subunits of the bacterial enzyme.

B. The mammalian bicistronic unit

The sequence of PHα57, a cDNA encoding the pig heart α-subunit (Chapter 5), was cloned into pT7 202 in place of the corresponding bacterial gene. The resulting plasmid, pT7 202/α57, now carried a hybrid system capable of producing pig heart α-subunit and *E. coli* β-subunit. Both subunits were efficiently

produced with this hybrid plasmid (Fig. 7.3*a*). Furthermore, the levels of accumulation of the two subunits were comparable to those seen with the *E. coli* subunits. Since the accumulation of the pig heart α-subunit paralleled that of the *E. coli* β-subunit, the translational coupling mechanism was still operating and had not been compromised by the presence of the foreign sequence.

The gene for the *E. coli* β-subunit in the hybrid plasmid was replaced by the corresponding pig heart sequence. The sequence where the two *E. coli* genes overlap was retained in this process. The plasmid, pT7 β/α57, now carries a bicistronic unit encoding the two pig heart subunits. Cells containing this plasmid continued to accumulate high levels of the α-subunit (Fig. 7.3*b*). The cells did not, however, accumulate similar levels of the pig heart β-subunit. The molecular weight of the pig heart subunit is 43 kDa and only minor increases in the amount of protein of this size were evident following induction. However, in addition to the α-subunit, a 28 kDa protein accumulated at high levels in the cells. The rate of accumulation of this 28 kDa protein was similar to that seen with the α-subunit. Cells carrying a plasmid with just the pig heart β-subunit sequence accumulated this 28 kDa protein and, as before, only minor amounts of the 42 kDa β-subunit were detected (Fig. 7.3*c*). Thus, the 28 kDa protein originated from within the β-subunit sequence.

Since production of the α-subunit depends on translation of the preceding sequence, either the β-subunit was synthesized at high rates and degraded rapidly or the translation of the α-subunit was coupled to the synthesis of the 28 kDa protein. Translation of the 28 kDa protein would, however, have to terminate close to the

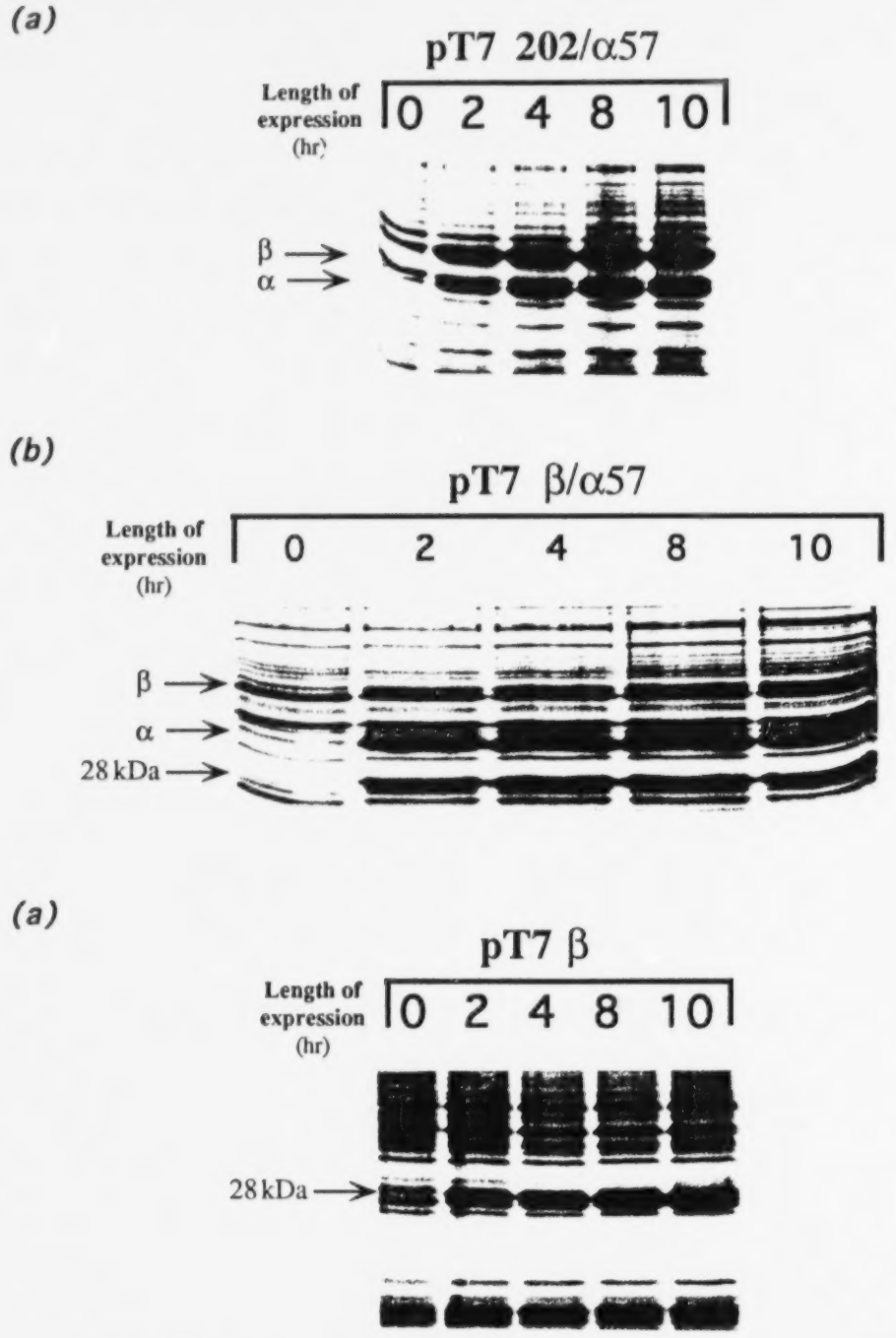


Figure 7.3. Expression of pig heart sequences in the bicistronic pT7 202 system. BL21(DE3) cells containing the expression plasmids, pT7 202/α57, pT7 β/α57 and pT7 β were grown in culture to an A_{600} of 0.5 and induced with IPTG. One ml samples were withdrawn at 0, 2, 4, 8 and 10 hours following induction, centrifuged and resolved by SDS-PAGE.

initiator of the α-subunit. The equivalence in the rates of accumulation of the 28 kDa protein and the α-subunit certainly supports the latter possibility. It is possible that the 28 kDa protein was the breakdown product of a β-subunit produced in high amounts.

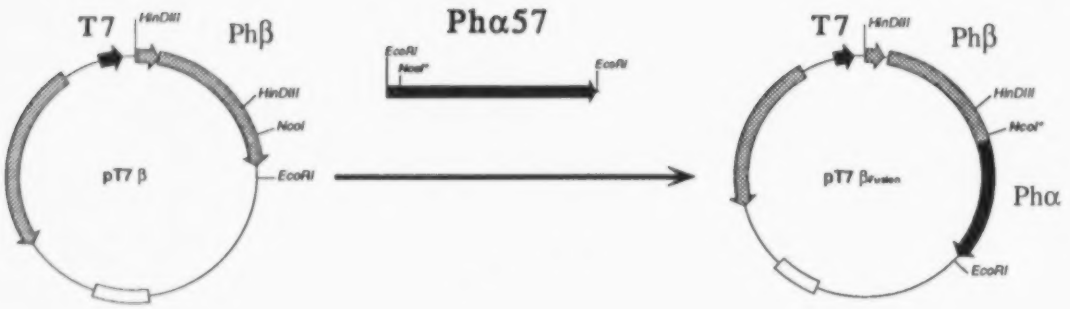
C. THE 28 kDa PROTEIN CORRESPONDS TO THE C-TERMINUS OF THE β-SUBUNIT

The sequence encoding almost the entire pig heart β-subunit was fused, in-frame, to the 5' end of the sequence encoding the pig heart α-subunit in pT7-6 (Fig. 7.4*a*). The expression of this fused sequence was examined. A 60 kDa protein accumulated in cells containing the sequence and no 28 kDa protein was produced (Fig. 7.4*b*). The size of the fusion protein produced would account for a 28 kDa protein fused to a 32 kDa α-subunit, suggesting that the 28 kDa protein represents a C-terminal fragment of the β-subunit. Furthermore, both the 28 kDa protein and the fusion protein were found to react strongly with antibodies directed against the pig heart enzyme. These proteins must, therefore, contain amino acid sequence from either the α- or β-subunit. If the β-subunit was specifically cleaved within the N-terminal part of its sequence to produce both the 28 kDa fragment and the 60 kDa fusion protein, then both these proteins should contain new N-terminal sequences from within the β-subunit.

D. THE 28 kDa PROTEIN IS TRANSLATED INDEPENDENT OF THE β-SUBUNIT

Both the 28 kDa fragment and the fusion protein were produced in *E. coli* as insoluble inclusion bodies (Fig. 7.4*b*). Thus, the proteins could be separated from most of the soluble host protein by

(a)



(b)

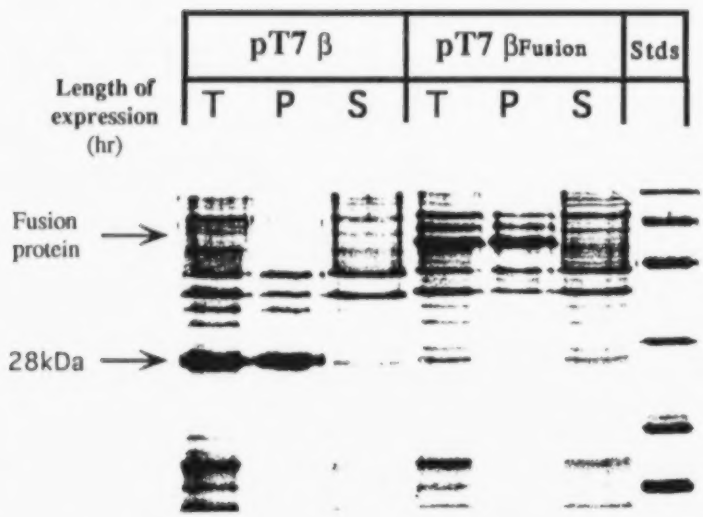


Figure 7.4. Analysis of fusion proteins of the pig heart β-subunit. ***(a)*** Plasmids were constructed for the production of the pig heart β-subunit on its own, pT7β, or as part of a fusion with the pig heart α-subunit, pT7βFusion. ***(b)*** BL21(DE3) cells containing these plasmids were grown up, induced for 5 hrs and samples were taken. Total cell lysates and fractions thereof (soluble **S** and pellet **P**) were analyzed by SDS-PAGE. Standards are 90, 66, 45, 30, 20 and 14 kDa in size.

centrifugation at 100xg. The 28 kDa fragment was purified in this manner, resolved on SDS-PAGE and transferred to PVDF membrane. The bound protein was cut from the remainder of the membrane and subjected to Edman degradation. The N-terminus of the 28 kDa protein contained a sequence identical in all but one residue to a stretch of amino acids within the β-subunit (Fig. 7.5*a*). Furthermore, the position of this sequence within the β-subunit was consistent with the size of the 28 kDa C-terminal fragment. The presence of methionine at the start of the N-terminal sequence was puzzling. Isoleucine is found in the corresponding position within the β-subunit. The nucleotide sequence just upstream of the codon for isoleucine resembles a bacterial ribosome binding site (Fig. 7.5*b*). Furthermore, the spacing between this ribosome binding site and the isoleucine codon is optimal for initiating translation. Initiation of translation from within the open-reading of foreign genes is frequently known to occur [23]. The ATT codon of isoleucine may have been used as an initiator, resulting in the incorporation of a methionine at the start of the 28 kDa protein. Use of ATT as an initiator has previously been reported for the *E. coli* initiation factor, IF-3 [24].

A restriction site just downstream from the authentic initiator codon for the β-subunit was cut, filled in and religated, resulting in a frame shift. Expression of this modified sequence continued to show accumulation of the 28 kDa protein (Fig. 7.5*b*). Thus, production of the 28 kDa protein did not depend on the synthesis of a β-subunit. The 28 kDa protein arose from an independent translation event. Translation from the initiator giving rise to the 28 kDa protein must

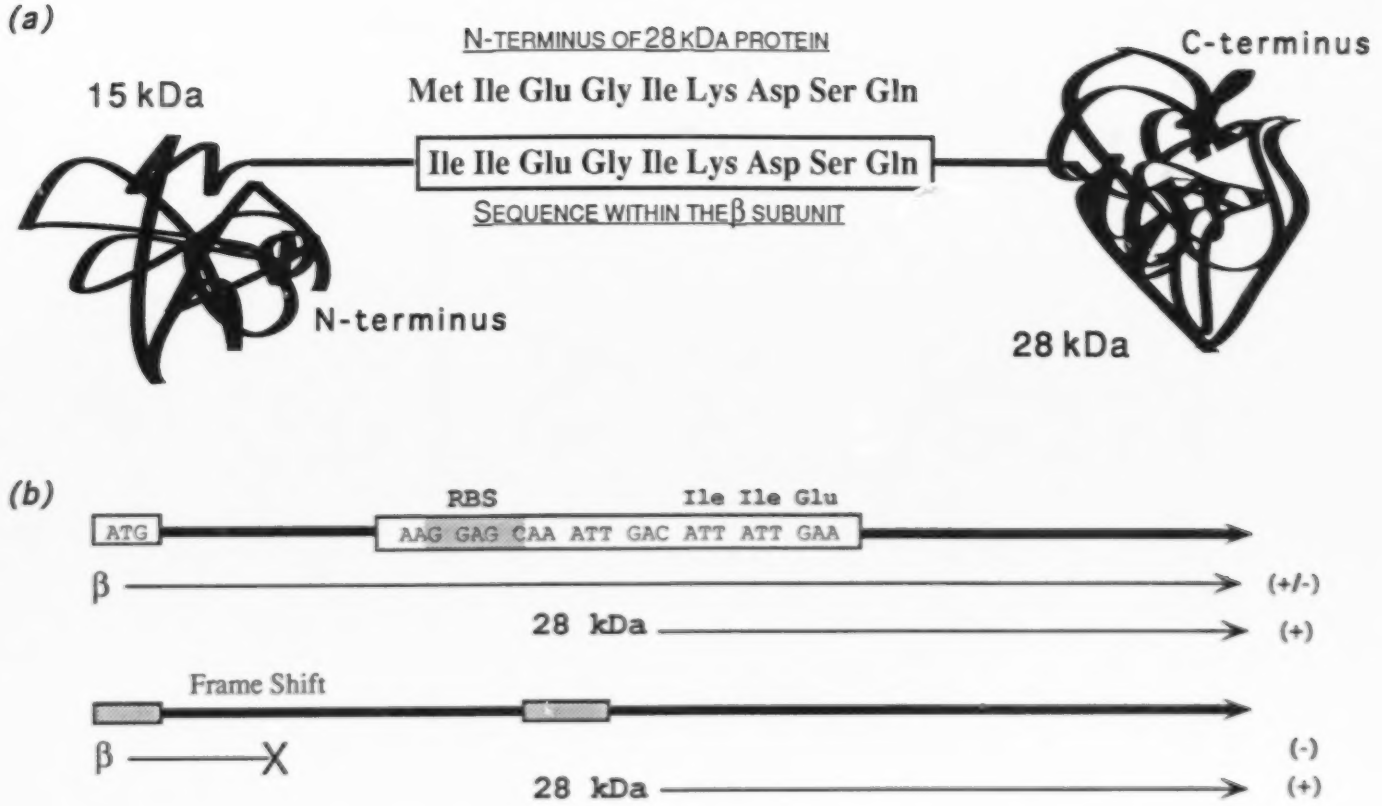


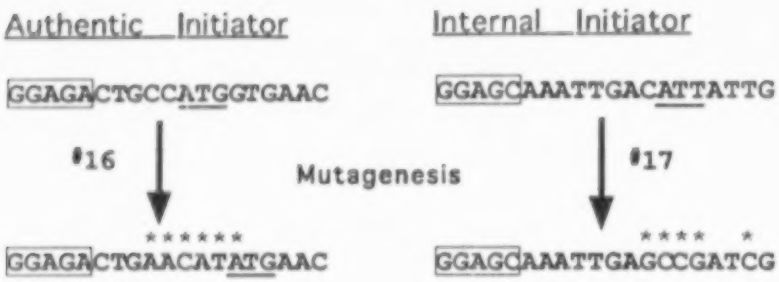
Figure 7.5. The 28 kDa protein arises by translation initiation from within the open reading frame for the β-subunit. ***(a)*** The N-terminus of the 28 kDa protein was determined by dansyl Edman degradation. The β-subunit primary sequence was searched for a match. The sequence with the highest identity from this search is shown. The predicted sizes of the two parts of β-subunit are 15 and 28 kDa. ***(b)*** Nucleotide sequence of the cDNA for the β-subunit highlighting the region encoding the N-terminus of the 28 kDa protein. The sequence contains a potential ribosome binding sequence (**RBS**) upstream from the ATT codon believed to be read as the initiator for the 28 kDa protein. The two possible translation products are depicted below the sequence. A sequence containing a frame shift upstream of the internal translation initiation site produces the 28 kDa protein alone.

have been very efficient since the levels at which this protein accumulates were high. Furthermore, the high levels of α-subunit produced with the 28 kDa protein in the cells containing pT7 β/α5 7 must have resulted from translational coupling to this internal initiation event.

E. Improving the translation initiation of the β-subunit

Alterations were made in the sequence of both the authentic and internal initiators to improve the synthesis of the complete β-subunit from pT7 β/α57. The initiator codon for the 28 kDa protein was altered (Fig. 7.6*a*). This change prevented the accumulation of the 28 kDa protein. Absence of the 28 kDa protein was accompanied by a dramatic drop in the levels of the α-subunit (Fig. 7.6*b*) demonstrating that synthesis of the α-subunit is dependent on translation coupling. The small amount of α-subunit produced may be a consequence of low levels of translation of the β-subunit. The sequence around the authentic initiator for production of the β-subunit was modified to increase its efficiency. The length of sequence between the ribosome binding site and the ATG was increased and its GC content was reduced (Fig. 7.6*a*). High levels of the β-subunit were produced with this modified sequence. In addition, higher levels of the α-subunit were found with this new sequence (Fig. 7.6*b*). This is likely a result of the combined coupling to two very efficient translation events upstream. When the two sequence alterations were combined, both subunits of the pig heart enzyme were produced in high levels in the absence of the 28 kDa protein (Fig. 7.6*b*). Moreover, the levels of accumulation of the two

(a)



(b)

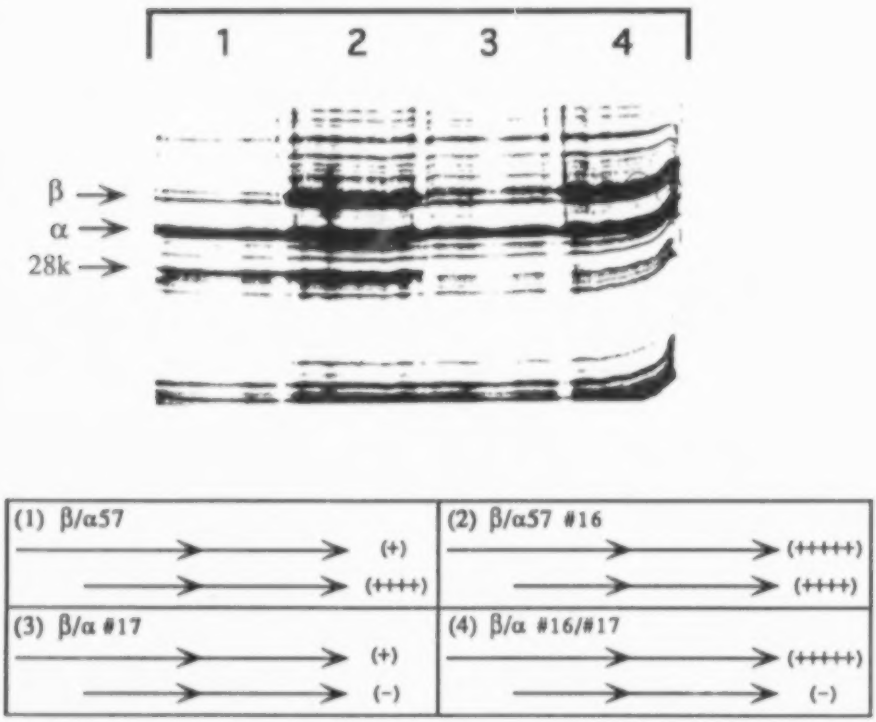


Figure 7.6. Mutagenesis of the two translation initiators and its effect on subunit levels. ***(a)*** Sequence alterations were made at the authentic and internal initiator sites. The sequence of the oligonucleotides, **#16** and **#17**, used to make these changes are given in **Appendix II**. An asterix identifies the residues that were changed. ***(b)*** The effect of these mutations on the levels of the α- and β-subunits and the 28 kDa protein. The results presented in lanes 1 to 4 are interpreted by their respective caption boxes below ((1) to (4)). Each box contains a pair of double arrows representing translation of the β- and α-subunit messages.

subunits were similar indicating that their syntheses were tightly coupled.

F. The effect of growth at room temperature

Despite these dramatic improvements in the production of the two subunits, no major increase in the enzyme activity of cell lysates was detected. Cells containing the expression vectors were induced for 5 hours, harvested and lysed by sonication. The lysates were separated by centrifugation into soluble and insoluble fractions and analyzed by SDS-PAGE. In each case the mammalian subunits were found in the insoluble fraction indicating that they had formed inclusion bodies (Fig. 7.7*a*). In contrast, the *E. coli* subunits were found primarily in a soluble form. A substantial amount of pig heart enzyme activity could be recovered by denaturing the expressed subunits in guanidinium chloride and refolding them according to established procedures [25]. Thus, the subunits present in the inclusion bodies are capable of reconstituting active enzyme.

It is general known that growth of bacterial cultures at low temperatures can often result in higher proportions of the expressed protein remaining soluble. Cells containing the pT7 β/α57 double mutant were grown at both 37 °C and 20 °C. Samples were withdrawn at various time points after induction was initiated. The enzyme activities were measured and compared. Cultures grown at 20 °C produced 20-fold higher levels of enzyme activity than those grown at 37 °C (Fig. 7.7*b*). Furthermore, up to 50 % of the subunits produced at 20 °C were found in a soluble form. Interestingly, the rates of appearance of enzyme activity in the two cultures were

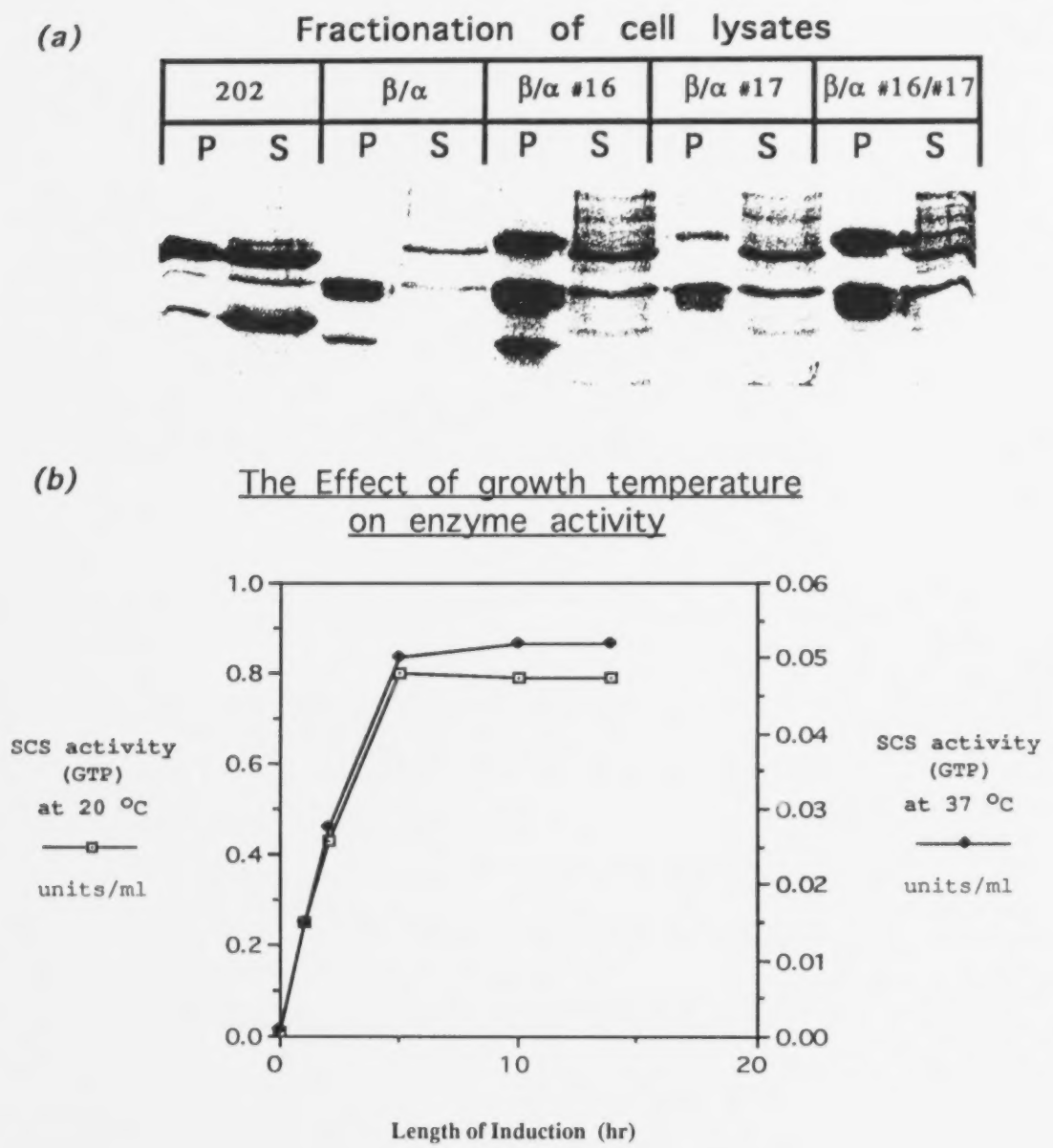


Figure 7.7. The effect of growth temperature on the solubility and yield of active enzyme ***(a)*** Cultures of cells expressing the different mutant sequences for the β-subunit were lysed by sonication and separated by centrifugation into supernatant and pellet fractions. Fractions were analyzed by SDS-PAGE. ***(b)*** Cells with the double mutant of the β-subunit sequence were expressed at 37^{0}C and at 20^{0}C. Samples of the cultures were removed at the indicated times following induction. The cells were lysed by sonication and the lysates assayed for SCS enzyme activity. GTP was used in the assays as the preferred substrate for the pig heart enzyme. Activity is given in units per ml of lysate. The levels of bacterial ATP activity were below 0.02 units/ml.

similar. A rapid increase in activity occurred within the first two hours after induction. This was followed by a slower increase up to the five hour time point, whereupon no further increases occured.

IV. DISCUSSION

The occurrence of a site for initiation of translation within the coding sequence of the pig heart β-subunit is not unique. Such internal translation initiation events are common when expressing eukaryotic genes in *E. coli* [23]. The source of this problem lies in the fact that eukaryotic sequences have evolved in the absence of selective pressures to reduce such sites from occurring within their code. Obviously, bacterial genes have evolved with reduced potential for internal initiation. Use of the isoleucine codon, ATT, as an initiator has previously been reported for the translation of the *E. coli* initiation factor, IF-3 [24]. Apart from problems with internal initiation, translation from the authentic initiator for the β-subunit was inefficient. Improvements in the efficiency of translation of the β-subunit have allowed the production of high levels of active pig heart enzyme. As a result, large amounts of the pig heart enzyme can now be produced for both structural and biochemical studies. Furthermore, the efficient expression of these sequences make rapid purification and analysis of mutants now possible.

The feasibility of using bacterial polycistronic translational coupling for the synthesis of two eukaryotic proteins has been well demonstrated here. In this case, a bacterial counterpart of the eukaryotic protein was available. The finding that translational

coupling can be achieved with foreign sequences demonstrates that its operation is independent of the sequences it couples. Synthesis of the two subunits of the pig heart enzyme was coupled as efficiently as that seen for the bacterial subunits. There are examples of sequences capable of translational coupling that do not involve the coding parts of genes [2,26]. This type of sequence could be used to couple any set of genes without having to modify it for each application. Multi-cloning sites could be placed on either side of the sequence and inserted into a vector generating a multi-use polycistronic expression plasmid.

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Section IV

Concluding Remarks

Chapter 8

Conclusions and future perspectives

I. CONCLUSIONS

A. Biogenesis and assembly

Pearson and Bridger [1] demonstrated that the *E. coli* enzyme could be reconstituted from mixtures of its constituent subunits. These reconstitutions were performed with subunits that had been prepared from the purified enzyme by denaturing gel filtration. Preparing the subunits in this manner is time consuming and requires large quantities of materials. Firstly, the enzyme must be purified, a process that involves several chromatographic steps and typically takes over one week. Separation of the subunits from the denatured enzyme by gel filtration is problematic because the molecular masses of the subunits are so similar. Consequently, this step results in losses and often needs to be performed twice.

A new procedure for the production and purification of the subunits is described in Chapter 2. The individual subunits of the enzyme were produced separately using a bacterial expression system. Both subunits accumulated as inclusion bodies in the bacterial cells. This greatly simplified their subsequent purification. The individual subunits could be prepared quickly and with a minimum of materials. These subunit preparations reconstituted active enzyme with efficiencies similar to those achieved with subunits separated from the purified enzyme. This new procedure provides a more versatile system with which to carry out further analyses of the assembly process.

The reconstitution experiments of Pearson and Bridger [1] showed the assembly of the *E. coli* enzyme was stimulated by the

presence of ATP. This effect was not seen with a non-hydrolyzable form of the nucleotide suggesting that it is the phosphorylation of the α-subunit that enhances the assembly process. However, a mutant of the *E. coli* enzyme in which the phosphorylated histidine was changed to an aspartate residue, assembled into a tetrameric complex resembling the native enzyme *in vivo* [2]. Furthermore, assembly of the mammalian enzyme is not effected by the presence of nucleotides [3]. Further investigations on the role of ATP in the assembly process could be carried out using the new reconstitution procedures in combination with mutagenesis.

The biogenesis of mammalian succinyl-CoA synthetase includes a step not found on the assembly pathway of the bacterial enzyme. Subunits of the mammalian enzyme are synthesized in the cytosol of the cell as precursors and must be imported into mitochondria prior to assembly [4]. The precursors contain N-terminally located signal sequences that are removed during the translocation process. The mature subunits are, then, released in the matrix where they assemble into the dimeric enzyme. Polypeptides requiring passage across biological membranes must be presented to the translocation machinery in a loosely folded conformation. Randall and Hardy [5] suggest that attainment of this translocation competent state is an intrinsic property of the precursor molecule, imparted on it by the presence of the signal peptide. The universal observation that signal sequences are removed from most translocated proteins suggests that their removal is a necessary prerequisite for folding and assembly to occur. This hypothesis was tested with precursor forms of the α-subunit of succinyl-CoA synthetase.

Precursors do not accumulate in eukaryotic cells under normal physiological conditions. Precursor forms of the α-subunit of succinyl-CoA synthetase were produced in bacterial cells. Like the mature subunits of the *E. coli* enzyme, these precursors accumulated in cells as insoluble inclusion bodies and were similarly purified. The folding, assembly and related properties of these precursors are described in Chapter 4.

Precursor forms of the α-subunit of both the *E. coli* and rat liver enzyme accumulated at significantly lower levels in bacterial cells than their corresponding mature forms. The lower levels of accumulation were the result of an increased sensitivity to proteolytic breakdown. Previous reports correlated the translocation competence of a precursor with the protein having a loosely folded, protease-sensitive structure [6]. It was, therefore, suggested that the precursor forms of the α-subunit had formed translocation competent conformations in the bacterial cells. In contrast to mature α-subunits, the purified precursors were compromised in their abilities to both phosphorylate and assemble with the mature β-subunit. This was seen as further evidence for the absence of a native-like conformation in the precursors. Together, these findings are consistent with reports in which precursors of several other mitochondrial proteins were found to adopt markedly different conformations from their mature counterparts [7,8]. The results add support to a model in which the signal sequence is solely responsible for the attainment of the translocation competent state of a precursor [5].

An alternative interpretation of these results would suggest that the signal sequence sterically hinders the α-subunit from either binding or hydrolyzing the nucleotide. Furthermore, the inappropriate positioning of a signal sequence could prevent the α-subunit from interacting with the β-subunit. The N-terminus of the mature α-subunit to which the signal sequence is attached is located some distance away from the surface that interacts with β-subunit. Moreover, it is the C-terminal domain of the α-subunit that carries the phosphorylated histidine. It is conceivable, though, that the signal sequence could extend from the N-terminus of the mature subunit down into the phosphorylation site as shown in Figure 8.1. Such positioning could easily interfere in both the phosphorylation of the subunit and assembly of the enzyme. If this interpretation was correct, then, the increased protease-sensitivity *in vivo* could be explained by the N-terminus of the signal sequence providing a more effective target for proteases.

Obviously, a more comprehensive study of the conformational state of mature and precursor forms of the α-subunit must be made to distinguish between these two models. Circular dichroism and tryptophan fluorescence measurements would provide good physical descriptions of the structures adopted by the different subunit forms. It would be of interest to examine the effect free signal sequences have on the folding of a mature subunit. In a similar vein, one might ask the question, do antibodies directed against the signal sequence overcome the interference in the folding of the subunit. Finally, demonstration that the precursor form of the α-subunit fails to phosphorylate does not distinguish between binding and

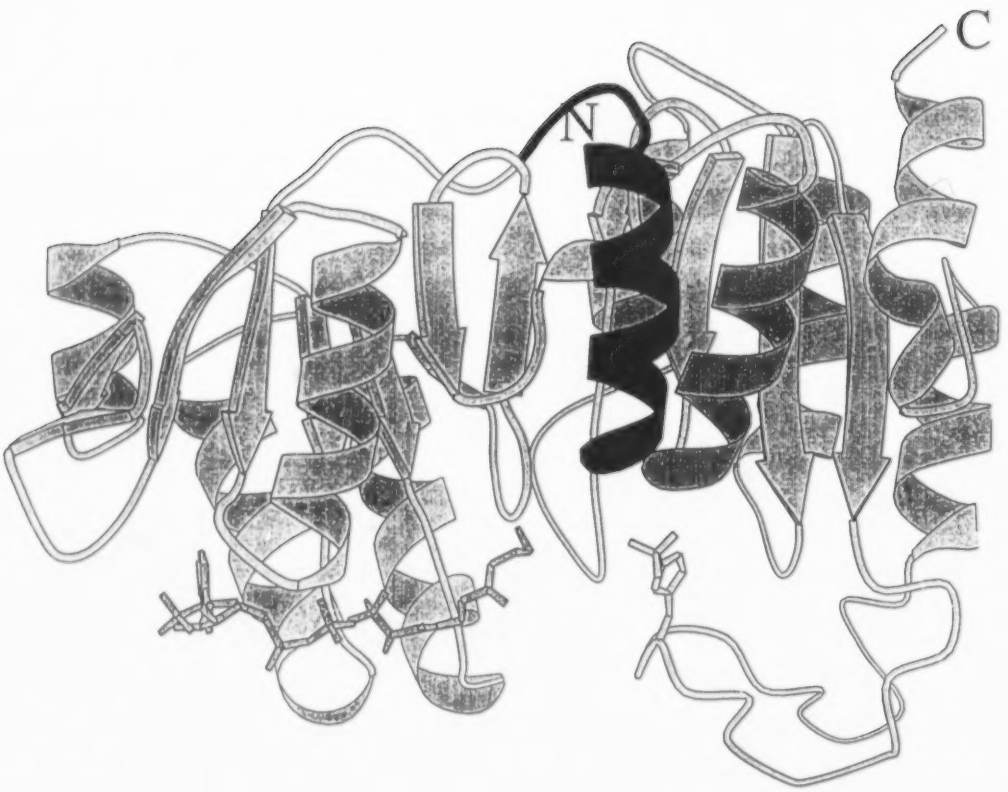

Figure 8.1. Model of a precursor form of the α-subunit of succinyl-CoA synthetase. A structural representation of the α-subunit is shown here with the signal sequence modelled into the structure as an α-helix (black). The position of the helix responsible for stabilizing the phosphohistidine is shown in grey tone. The graphic depicting the α-subunit was taken from Wolodko *et al.*, 1994 [8].

hydrolysis of the nucleotide. To address this question, nucleotide binding studies need to be carried out with the precursor. Such binding studies could be complemented by fluorescence quenching data.

B. Mammalian isoforms

In addition to its position within the tricarboxylic acid (TCA) cycle, mammalian succinyl-CoA synthetase plays a key role in ketone body catabolism and porphyrin synthesis. In the TCA cycle, succinyl-CoA is converted to succinate, whereas ketone body breakdown requires high levels of succinyl-CoA to be maintained [9]. It has been suggested that such opposing functional demands on the enzyme-catalyzed reaction could best be accommodated by two distinct enzymes [10]. Reports of isoforms of the mammalian enzyme support this concept. Separate ATP- and GTP-specific enzymes were found in tissues from a wide range of animal species including mammals [10,11]. The isolation of cDNAs encoding two forms of the α-subunit of pig heart succinyl-CoA synthetase described in Chapter 5 is consistent with these reports.

One of the cDNAs encoding the α-subunit of the pig heart enzyme, PHα108, contained a unique stretch of 108 nucleotides within its open reading frame that replaced a shorter 57 nucleotide section in PHα57. The mRNAs that gave rise to the two pig heart clones originated from a single gene and were the result of a mutually exclusive splicing event. The generation of the PHα108 message involves atypical donor and acceptor splice sites. The dinucleotide AT is found in place of the preferred GT [12] at positions

+1 and +2 of its donor splice site. However, five of the first six nucleotides at the 5' end of the intron are identical to the consensus sequence for a donor splice site [13]. It is suggested that such adherence to the remainder of the consensus may make use of this site possible. Both the 57 and the 108 sequence are spliced to a common 3' acceptor site. This site is being differentially utilized by the two sequences. The 108 sequence uses the dinucleotide AA rather than the preferred AG used by the 57 sequence at the this site. Interestingly, all cases where non-consensus acceptor sites are used involve differential splice site selection [14]. Such differential use of this 3' splice site might represent an important control point regulating the relative levels of two mRNA species.

The PHα57 message would be expected to predominate since it follows the more accepted rules for splicing. The observation that 22 out of the 25 cDNAs characterized contained the 57 sequence support this viewpoint. However, the relative abundance of the two mRNAs need to be determined by more direct methods. Preliminary Northern blot analysis does show evidence of two transcripts, 1.8 kb and 1.6 kb in size. It has yet to be determined whether either of these species contains the unique 108 sequence. The longest cDNA obtained in this study was 1.3 kb and contained just 42 nucleotides of 5' non-coding sequence. Since the 3' end of this cDNA contained a polyA tail, the lengths of the transcripts detected in Northern blots indicate that the 5' untranslated region of the cDNA is incomplete. Three genomic clones, spanning ~35 kb of the genome, were isolated in this study. It is likely that the entire α-subunit gene is contained within these clones. Future studies directed toward elucidating the

structure of the promoter for this gene would be of special interest given the variety of metabolic roles proposed for the enzyme.

In Chapter 6 the two cDNAs, PHα57 and PHα108, were combined with a cDNA encoding the β-subunit of the pig heart enzyme [15] and expressed in bacterial cells. Both of these combinations produced a GTP-specific enzyme. Extracts of the bacterial cells containing the two enzymes were fractionated by hydroxyapatite chromatography. The isoform produced by the β/α57 combination displayed an elution behaviour typical of that seen with the enzyme purified from pig hearts [16]. Moreover, the α-subunit produced by the PHα57 cDNA is similar in size to the corresponding subunit from the purified enzyme [17]. The behaviour of the β/α108 isoform on hydroxyapatite was significantly different from that seen with the β/α57 enzyme. The β/α108 enzyme eluted from the column at a phosphate concentration that was lower than that required to elute the β/α57 enzyme. Thus, hydroxyapatite chromatography is capable of distinguishing between these two forms of pig heart succinyl-CoA synthetase

Two distinct GTP-specific enzymes were seen in mouse liver [18]. The two forms could be distinguished by their pH profiles and by their behavior to cellulose acetate electrophoresis. One of these enzyme forms was elevated threefold under conditions of increased porphyrin synthesis. It was suggested that a GTP-specific enzyme, operating under the high GTP/GDP ratios in mitochondria would maintain favorably levels of succinyl-CoA for ketone body metabolism [19]. In support of this, Jenkins and Weitzman [20] showed that the level of GTP-specific activity was significantly

increased in tissues of ketotic animals. These reports highlight the role of GTP-specific enzymes in both ketone body metabolism and porphyrin synthesis. The demonstration of two distinct of GTP-specific isoforms of succinyl-CoA synthetase may be relevant to these proposals.

It was noticed that the behaviour of the β/α108 enzyme on hydroxyapatite was strikingly similar to a GTP-specific succinyl-CoA synthetase activity previously identified by Cha and colleagues [16]. They reported finding a minor peak of enzyme activity eluting from their columns at a phosphate concentration similar to that at which the β/α108 enzyme eluted. A preparation of the enzyme that had been purified from the heart tissue of a one week old piglet was subjected to hydroxyapatite chromatography. Fractions eluting from the column at the phosphate concentration at which the β/α108 form emerges were found to contain this minor peak of enzyme activity. Furthermore, antibodies directed against the unique portion of the α108-subunit immuno-reacted to a protein contained in these fractions. These findings strongly suggest the presence of a α108-related protein in pig heart tissue. Using the information gained in these studies, it should be possible to purify this α108-related isoform from mammalian tissues in amounts sufficient for further biochemical analysis.

The amino acid sequence encoded by the 57 nucleotide stretch of PHα57 is conserved in the α-subunits of the *E. coli* [21], *Thermus flavus* [22] and *S. cerevisiae* [23]. The position of this sequence in the x-ray crystal structure of the of *E. coli* enzyme[24] is illustrated in Figure 8.2*a*. The sequence forms an interconnecting structure

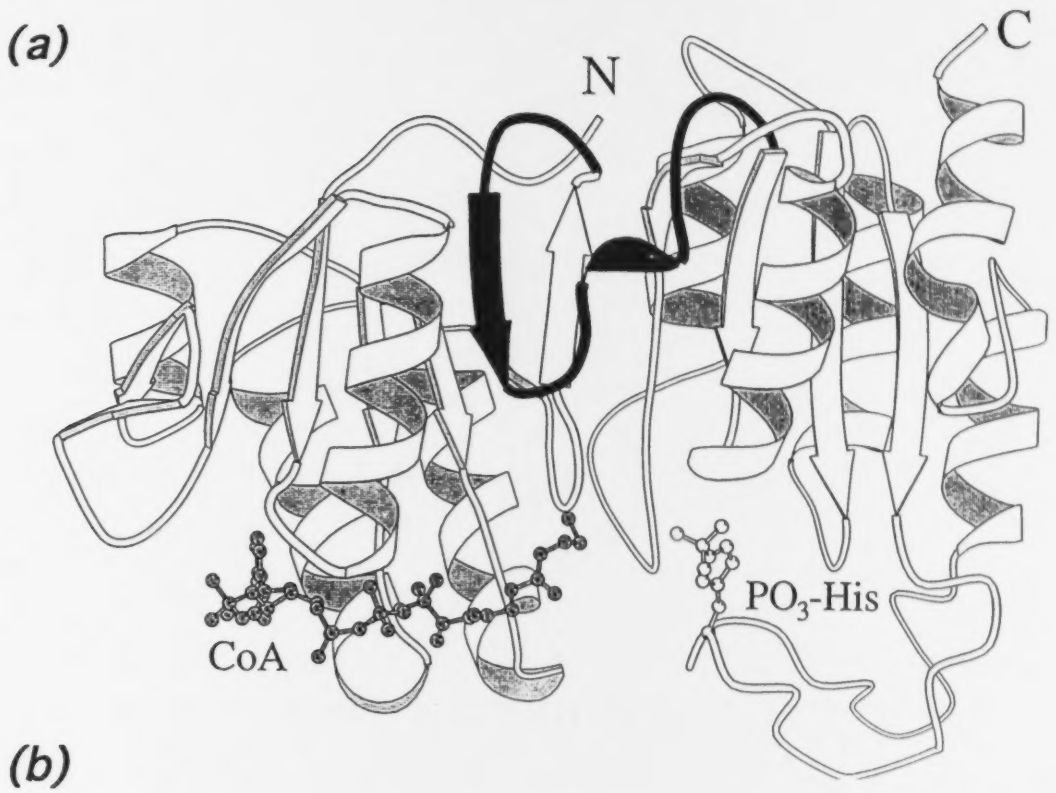

Figure 8.2. Position of the alternatively spliced sequence in the α-subunit. ***(a)*** Ribbon diagram of the structure of the α-subunit of *E. coli* succinyl-CoA synthetase. The 57 encoded sequence is shown in black. ***(b)*** Alignment of the 57 encoded sequence and surrounding residues of several α-subunits. ATP citrate lyase is included for comparison. Shaded boxes highlight residues showing identity in the lyase and α-subunit sequences.

between the two domains of the α-subunit, the N-domain responsible for binding CoA and the C-domain which contains the phosphohistidine residue. The amino acid residues on either side of this sequence are highly conserved (Fig. 8.2*b*). The residues, SRSGTLTYE, lying next to the C-terminal end of the sequence are identical in all α-subunits examined. These residues form an α-helix within the phosphorylation domain and is implicated in stabilizing the phosphohistidine [24]. Residues next to the N-terminal end of the sequence form a loop that comes in close contact with the thiol group of CoA.

The reaction catalyzed by the enzyme, ATP-citrate lyase, is very similar to that of succinyl-CoA synthetase and also involves the intermediate phosphorylation of a histidine residue. Part of the ATP-citrate lyase molecule shows homology to the α-subunit of succinyl-CoA synthetase [25]. Interestingly, the region in ATP-citrate lyase corresponding to the 57 encoded sequence differs and its size is larger due to the insertion of an additional eight amino acid residues. The three dimensional structure illustrates the adaptability of this region of the protein for accommodation of larger sequences. It is, thus, conceivable that a sequence such as the one encoded by PHα108, although twice the size of the 57 encoded sequence, could fit structurally within the α-subunit.

C. Expression of the mammalian sequences in *E. coli*

Several useful lessons have been learned with regard to the expression of foreign sequences in *E. coli*. Firstly, the 5' end of foreign sequences must be optimized so as not to interfere with the

structure and operation of the translation initiation region [26]. This fact is very well illustrated by the results presented in Chapter 3 wherein the precursor form of the rat liver α-subunit failed to accumulate in bacterial cells. Initiation of translation was found to be adversely affected by the proximity of the 5' end of the sequence to the translation initiation region. Insertion of a leader sequence between the ATG initiator and the start of the cDNA reading frame allowed the efficient production of a precursor form of the subunit. It is suggested that an effective and general strategy to circumvent this problem would be to produce the foreign protein as a fusion protein.

The second point to be made is that translation initiation can often occur from within the coding region of a foreign sequence expressed in *E. coli* [27]. Such an event can have drastic consequences for the production of the full length protein as seen with the β-subunit of the pig heart enzyme in Chapter 7. It was pointed out that eukaryotic mRNAs have evolved without the need to prevent such sequence from occurring. Obviously, no general strategy can be proposed for dealing with such problems. Vigilance is advised.

Finally, a system capable of coordinating the production of the two subunits of the pig heart enzyme has been described in Chapter 7. This system makes use of the translational coupling mechanism employed to coordinated the production of the corresponding bacterial subunits [21]. It is suggested that this method may be more generally applied for coordinating the production of a variety of enzyme activities in bacteria. The subunits of the pig heart enzyme were found to be insoluble when produced

at 37 °C. Production of the subunits at 20 °C alleviated this problem. As a result, large quantities of mammalian succinyl-CoA synthetase can now be produced. This will allow a more detailed analyses of the structure and mechanism of the eukaryotic enzyme to be made.

II. FUTURE PERSPECTIVES

Elucidating the reaction mechanism at the molecular level promises to be the most interesting area of research on this enzyme in the near future. Mutagenesis studies have been considered ever since the *E. coli* genes became available. Expression of the genes in a bacterial system was optimized [28] making follow-up biochemical and structural analyses feasibly. As more and more sequences began to appear in the literature, comparisons were made and strong sequence identity was found. This was especially evident in the α-subunit (Chapter 1, Fig. 1.6). With the structural information at hand by 1993 [24], a direct attack on molecular reaction was initiated.

Phosphorylation of the histidine residue is envisioned as a nucleophilic attack by the N-3 atom on the terminal phosphoryl group of ATP or on the phosphoryl group of succinyl phosphate. Since the tautomeric form of the imidazole ring containing the lone pair of electrons on the N-1 is favored, it has been suggested that the tautomer with the lone pair at the N-3 is stabilized by an anionic group [29]. In the structure, αGlu_{208} is ideally positioned to play this role (Fig. 8.3). The carboxylate group of αGlu_{208} in the C-terminal domain of the α-subunit interacts with the imidazole ring of

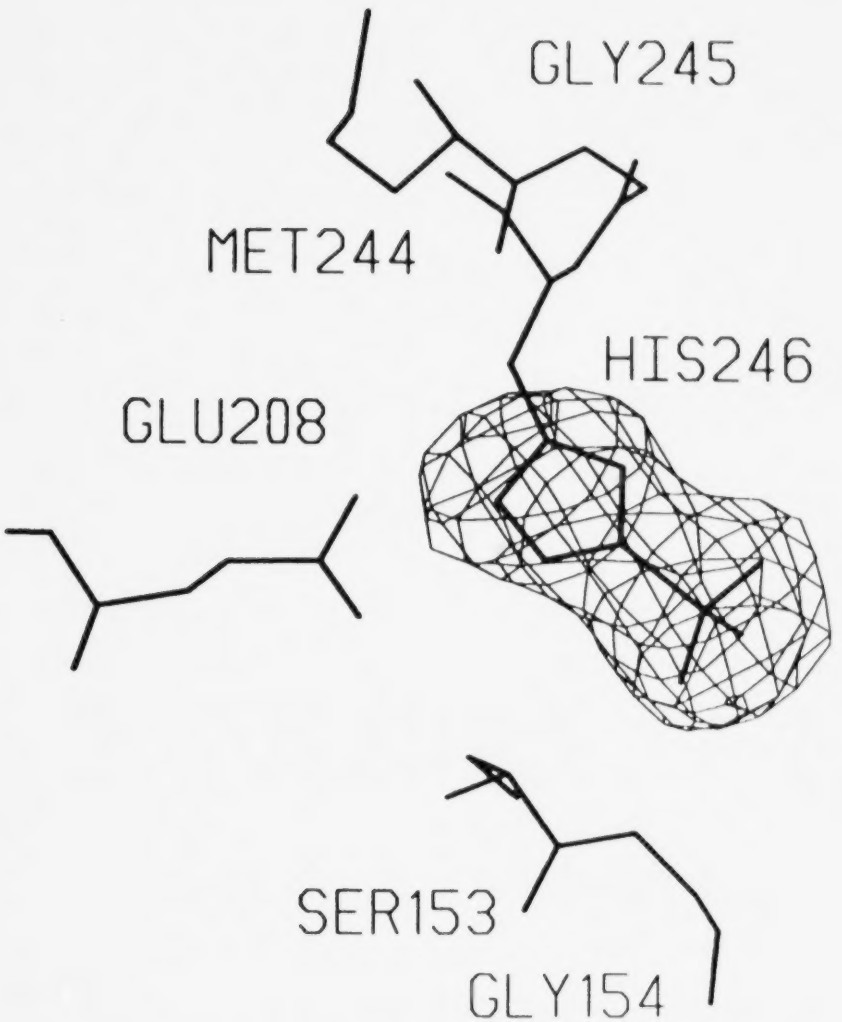

Figure 8.3. Part of the active site of *E. coli* succinyl-CoA synthetase. A structural representation of part of the active site of succinyl-CoA synthetase illustrating the positions of residues close to the phosphorylated histidine. All of the residues shown here are from the α-subunit of the enzyme. This graphic was taken from Wolodko *et al.*, 1994 [8].

phosphohistidine at the N-1 position. αGlu_{208} is located on a loop connecting two secondary structures. The amino acid sequence of this loop is identical not only in the α-subunits but also in the aligned ATP citrate lyase (Chapter 1, Fig. 1.6 *b*). αGlu_{208} was replaced with aspartate, glutamine and alanine in the *E. coli* enzyme (Ryan *et al.*, unpublished work). The $\alpha Glu_{208}Asp$ mutant retained full catalytic activity. However, both $\alpha Glu_{208}Gln$ and $\alpha Glu_{208}Ala$ suffered complete loss of activity indicating that removal of the carboxylate group inactivates the enzyme. This is consistent with the proposed role of the glutamate residue. Furthermore, since $\alpha Glu_{208}Asp$ is unaffected by the change, there must be sufficient mobility in the loop to position the carboxylate of the shorter aspartate side chain next to the N-1 atom of the histidine residue.

Two oxygen molecules of the phosphoryl group of phosphohistidine are situated close to the N-termini of two α-helices and are coincident with the helix axis of both (Chapter 1, Fig. 1.8 *b*). It is believed that the partial positive charges at the N-termini of these helices serve to stabilize the phosphohistidine residue. This may explain the unusually stable nature of the phosphoform of the enzyme [30]. The amino acid sequence of both of these helices are extremely well conserved. The helix that is part of the C-terminal domain of the α-subunit is comprised of the longest stretch of conserved residues in the protein (Chapter 1, Fig. 1.6 *c*).The side chain hydroxyl groups of αSer_{153} and αThr_{155} and the amide nitrogen of αGly_{154} are within hydrogen-bonding distances of one of the oxygens (Fig. 8.3). αSer_{153} and αThr_{155} were replaced with alanine residues in the *E. coli* enzyme (Ryan *et al.*, unpublished work). The

$\alpha Ser_{153}Ala$ replacement caused a dramatic reduction in activity illustrating the importance of this residue at the active site. The specific activity of the mutant enzyme was reduced one hundred-fold. The $\alpha Thr_{155}Ala$ substitution still retained 15 % of the original activity. Further work on these and other residues within the active site is currently in progress.

In conclusion, combining x-ray crystal structure information for the enzyme with the ability to carry out mutagenesis will provide a very good framework for future exploration. Knowledge gained from such an approach is already providing new answers to old questions.

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Appendices

I. BACTERIAL STRAINS AND GENOTYPES

Strain	Genotype	Reference
BL21(DE3)	*hsdS gal* (λcI*ts*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene *1*	[1]
CJ236	*dut*1 *ung*1 *thi*-1 *relA*1/pCJ105(*cam*^{r} F')	[2]
DHI	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	[3]
GM48	*thr leu thi lacY galK galT ara tonA tsx dam dcm supE*44	[4]
JM109	*recA*1 *supE*44 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	[4]
LE392	*mcrA hsdR*514 *supE*44 *supF*58 *lacY*1 *galK*2 *galT*22 *metB*1 *trpR*55	[5]
TK3D18	Δ(*kdp-suc*)D18 Δ(*gal-bio*)	[6]
Y1089	Δ*lac*U169 Δ*lon araD*139 *strA hflA*150::Tn*10*(*tet*^{r}) [pMC9 *amp*^{r} *tet*^{r}]	[7]

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II. OLIGONUCLEOTIDES

#1. GGAGGGGAAACATATGTCCATTTTAATCG

#2. GAAAAATATCTATATCTAGAAAAATACG

#3. GAAAAATATCTCCATGGAGAAAAATACG

#4. GAAAAATATCTATATCGATAAAAATACG

#5. CACAGGCTGCTGCGCCAGGGAAA

#6. CCAACTTGTGTTGTTTGATGAACTGC

#7. AATGCAAGCTTGGCATCATGCCTGGC

#8. CTTCCTGTATTCTTCCTGCTG

#9. TGAATATGGCCAGGCATGATG

#10. GTGCATGCTGGAACCAGAAGTA

#11. CATCTCTATATCGATAAAAATACG

#12. GGACTGCCATGGTGAACCTGCAGG

#13. GGCCACAGCCTGCTGAGCTGCATCCTC

#14. GCCAGCCTGATCTGTGACATATGCGTGACCAG

#15. GGAGGGGAAACATATGTCCATTTTAATCG

#16. AGACTGAACATATGAACCTGCAGGA

#17. GAGCAAATTGAGCCGATCGAAGGAATAAAG

III. MEDIA PREPARATION

LB Medium

Bacto tryptone	10g
Bacto yeast extract	5g
NaCl	10g
H_2O	1 Liter

LB/KCl Medium

Bacto tryptone	10g
Bacto yeast extract	5g
NaCl	5g
KCl	5g
H_2O	1 litre

M9 Medium

10 x salt base*	100ml
20 % glucose	20ml
10 mM $CaCl_2$	10ml
100 mM $MgSO_4$	10ml
H_2O	860ml

10 x Salt base*

Na_2HPO_4	70g
KH_2PO_4	30g
NaCl	5g
NH_4Cl	10g
H_2O	1 Liter

IV. CHROMATOGRAPHY

Sephacryl S300 and S200 (Pharmacia)

Resin is swollen in 50 mM potassium phosphate (pH 7.4), 1 mM EDTA. Columns, 120 cm in length and 1.25 cm in diameter, are packed with the swollen resin under gravity. The columns are washed with five volumes of phosphate buffer. Samples of up to 5 % of the column volume are gently layered over the top of the resin. The columns are run with 50 mM potassium phosphate (pH 7.4), 1 mM DTT, 1 mM EDTA.

Affigel Blue (Biorad)

Resin is swollen in 50 mM potassium phosphate (pH 7.4), 1 mM EDTA. 50 ml columns, 20 cm in length and 4 cm in diameter, are packed with the swollen resin under gravity. The columns are washed with five volumes of phosphate buffer. Samples are applied to the column in 50 mM potassium phosphate (pH 7.4), 1 mM DTT, 1 mM EDTA. Bound protein is eluted from the column using a 0-2 M NaCl gradient.

Hydroxyapatite (Biorad)

Resin is swollen in 10 mM potassium phosphate (pH 7.4), 1 mM EDTA. 25 ml columns, 10 cm in length and 4 cm in diameter, are packed with the swollen resin under gravity. The columns are washed with ten volumes of phosphate buffer. Samples are applied to the column in 10 mM potassium phosphate (pH 7.4), 1 mM DTT, 1 mM EDTA. Bound protein is eluted from the column using a 10-400 mM potassium phosphate gradient.